Development of an High Performance Liquid Chromatography-Mass Spectrometry Method to Determine Ferulic Acid Oligomers in Plant Materials

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Chapter 1 Introduction and Study Objectives

1.1 Introduction

Plant cell walls are primarily composed of polysaccharides suspended in either pectin or arabinoxylan networks with lignin and structural proteins present to a lesser extent. Hydroxycinnamic acids (HCAs), which are minor constituents in plant materials, are involved in the cross-linking of plant cell wall components through oxidative coupling. Oxidative coupling of HCAs results in the formation of hydroxycinnamic acid dimers and potentially higher oligomers. The formation of dimers, trimers, and tetramers was described for ferulic acid, which is the dominant HCA in grasses such as cereals. Ferulic acid cross-links are important for the development of plant cells since they are involved in the cessation of cell elongation and stiffening and strengthening of the plant cell wall. These physiochemical changes in the plant cell wall are also involved in plant resistance to fungi and insects, have an impact on textural changes in fruits and vegetables and on the physiochemical and, potentially, physiological properties associated with dietary fiber. Despite their importance in many disciplines the knowledge about ferulic acid dimers and especially oligomers in plant materials is rather limited. These compounds have proven difficult to study and quantify for several reasons. HCAs, and in particular oligomers of ferulic acid, occur in very small quantities in plant materials. They exist as different regiosisomers, multiplying the number of analytes. In addition, it is hard to obtain these compounds as standard materials since they are hard to synthesize and, due to their low abundance, laborious to isolate. As such, many quantitative methods currently in use have not been validated. This has led to the incorrect estimations of

ferulate dimer and trimer compositions and levels. Therefore, the aim of this study was to improve the detection and quantification of dehydrodiferulic acids (dFAs) and dehydrotriferulic acids (tFAs) in plant materials.

1.2 Study Objectives

To improve analytical procedures to identify and quantify ferulic acid dimers and trimers in plant materials the following study objectives were defined:

- Isolate dFAs and tFAs from maize bran for use as standard compounds
- Develop a gas chromatography and/or liquid chromatography mass spectrometry method to quantify dFAs and tFAs in plant materials
- Perform a validation of the developed methodology
- Apply the developed methodology to plant materials

Chapter 2 Literature Review

2.1 Nature and Occurrence of Ferulic Acid Oligomers and Their Impact on the Plant Cell Wall and Plant Based Food Products

HCAs are phenolic compounds ubiquitously occurring in plants in minor amounts. They are formed in the phenylpropanoid pathway from the aromatic amino acids phenylalanine and, in grasses, tyrosine. Although they can be found in their free form or attached to low molecular weight compounds (e.g. chlorogenic acids in coffee), they are primarily connected to plant cell wall components, especially in grasses [1]. HCAs attached to the cell wall polymers are ferulic acid (FA), *p*-coumaric acid (CA), and sinapic acid (SA) [2]. These compounds have the same basic structure composed of a phenolic ring with a propenoic acid attached at the C1 position but differ in the amount and location of methoxy groups attached (**Figure 1**).

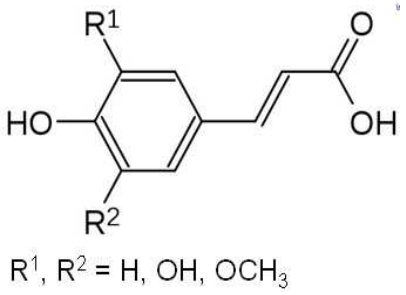

Figure 1. Hydroxycinnamic acids. R1=H, R2=H, p-coumaric acid; R1=H, R2=OCH3, ferulic acid; R1=OCH3, R2=OCH3, sinapic acid, R1=H, R2=OH, caffeic acid.

The HCAs are biosynthesized in their *trans*-configuration, however, UV-light can partially convert them into their *cis*-isomers. In grasses, FA is commonly ester-linked to arabinoxylans, CA to lignin and arabinoxylans and SA to polysaccharides (potentially arabinoxylans). Less is known about the attachment of HCAs to cell wall polymers in dicots but FA seems to be primarily ester-linked to pectic arabinans and galactans [3]. Due to their phenolic structure, HCAs can readily be oxidized by peroxidases and oxidases, such as laccase, forming a radical. This radical can be stabilized by mesomerism around the phenolic ring and along the propenoic acid side chain. Coupling of two radicals leads to the formation of both homo- (coupling of HCAs of the same type) and heterodimers (coupling of different HCAs) (**Figure 2**).

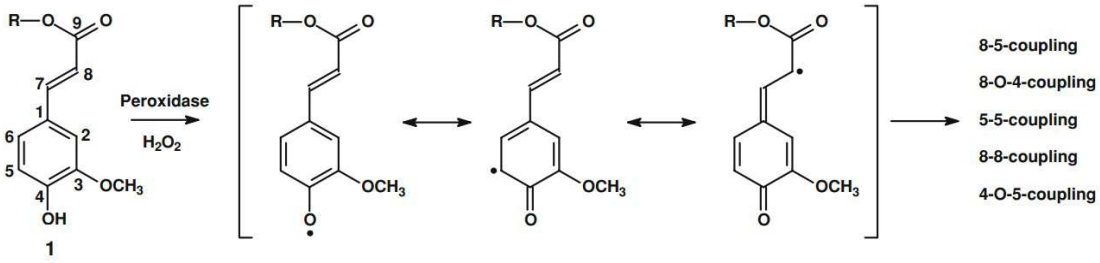

Figure 2. Formation of a ferulate phenoxyradical by the action of peroxidase/H_2O_2 and its resonance stabilization. Coupling of two ferulate radicals can theoretically lead to 8-5, 8-O-4, 5-5, 8-8 and 4-O-5 coupling [1].

Most commonly found are homodimers, especially dFAs. Depending on the position of the radical during the coupling reaction, several regiosiomers can be formed, i.e. 8-5-, 8-8, 8-O-4, 5-5- and 4-O-5-coupled dimers. Further radical coupling leads to the formation of tFAs and higher oligomers (**Figure 3**).

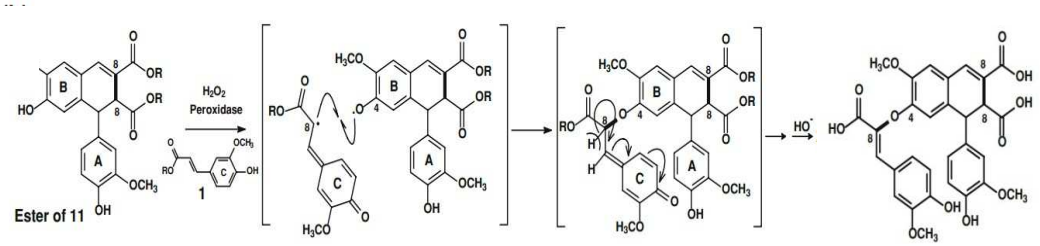

Figure 3. Formation of 8-8cyclic/8-O-4-dehydrotriferulic acid through radical coupling reactions [1].

This di- and oligomerization links HCAs to each other thereby cross-linking plant cell wall polysaccharides. Other oxidative coupling reactions of HCAs can also cross-link polysaccharides to lignin and proteins.

Cross-linking results in changes to the physicochemical properties of the plant cell wall which impacts many areas of academic and commercial research. In general, chemical reactions between cell wall components are often directly related to the quality of plants as food products [4]. For example, the formation of dFA cross-links has been associated to the tensile strength of beet root cell walls and the crispness and cell wall stiffening associated with Chinese water chestnuts [5]. On the other hand, the loss of dFA cross-links has been shown to correlate with the loss of tissue strength in plant materials which is a problem in post-harvest softening. dFA cross-links are also of interest for processed food products. These compounds potentially affect the amount of water extractable arabinoxylans (WEAX) in wheat and rye breads [6]. The cross-linking of xylans by oxidatively formed dFAs (and potentially oligomers) has also been discussed to affect the rheological properties of oat and wheat dough [7]. In addition, FA has been found cross-coupled to tyrosine potentially forming a phenolic-protein complex in bread [8].

Oxidative cross-linking of feruloylated polysaccharides can also lead to gel formation of plant materials. Beet pectin was oxidatively cross-linked leading to gel formation [9]. dFA contents in the gel were higher than in the starting material. However, the decrease of monomeric ferulates in the gel could not be explained by the formation of dFAs only. The authors therefore speculated that higher oligomers than dimers are involved in pectin cross-linking and gel formation, too. tFAs and potentially higher oligomers of FA were also suggested to be involved in the WEAX gel formation [10]. The formation of pectin or arabinoxylan gels is not only important from a food processing point of view but may also have an impact on the health benefits of these products. The formation of gels has been associated with the lowering of post-prandial blood glucose levels [11]. Other health benefits of dFAs may be due to their antioxidant activity. 8-O-4-dFA was shown to have higher antioxidant activity than ferulic acid itself and the main antioxidant mechanism of dFAs was determined to be primarily as a radical scavenger [12, 13]. Although 8-8-coupled dimers can also chelate metal ions. Next to their antioxidant activity, dFAs and their microbial metabolites have been demonstrated to have anti-inflammatory properties in cell cultures [14]. Finally, dFAs may act as antimutagens in the human body [15]. From a plant physiological point of view, increases in dFA content have been observed with decreases in cell wall elongation and a corresponding increase in the relaxation rate of the cells [16]. More specifically, 5-5-dFA has been associated with the cessation of cell elongation in internodes of floating rice [17] and interruption of dFA cross-linking has even been shown to increase cell elongation in oat leaves [18]. These changes in cell wall structure can impact fungal and insect resistance of plants, too. Higher levels of dFA cross-linking was found to be instrumental in lending plants resistance to *Fusarium*

graminerium (the causative agent of Gibberella stalk rot), the Mediterranean corn borer, and to the maize weevil [19-22]. Resistance to these fungi and insects may be due to the prevention of the initial plant wound through which the invasive organism enters or the inability of the disease agent to digest the plants tissues (Bunzel, 2010). In addition to their contribution to resistance to *F. graminerium,* dFAs have been shown to protect plants from *Fusarium culmorum* by inhibiting the production of the type B trichothecene toxin [23].

Although desirable in many fields, increase in the strength of the cell wall materials is not always wanted. It can, for example, negatively impact forage digestibility. This can be due to the inability of the fungal enzymes to digest the material (Grabber, 1998) because of dFA mediated cross-linking of arabinoxylans and lignin in some forage materials [24]. Accordingly, recent work has shown that ferulic acid esterase can be used to improve the digestibility of tall fescue grass as a forage material [25]. The involvement of HCAs in plant cell wall structure is so important that they are often used in biomass acceptance screening for potential bioenergy crops [26].

2.2 Analytical Aspects of Hydroxycinnamic Acids and Ferulic Acid Oligomers

Identification and quantification of HCAs and FA oligomers are critical to better understand the processes in which they are involved. The ability to screen both plant materials and processed plant based products for their HCA, dFA and tFA contents provides important information about potential chemical reactions, reactions that may have occurred, or if the plant could be a useful source of certain phytochemicals.

For example, identification and quantitation of dFAs, tFAs and higher oligomers provides essential information about the extent and nature of the cross-linking of plant materials. It

further supports localization of cross-links in the plant organs and tissues and how cross-links affect tissue maturing, plant growth etc. As HCAs and their oligomers become more important and their physiological effect understood it will be important to understand the distribution of these compounds within plant materials. Identification and quantitation of different FA oligomers not only contributes to the body of knowledge of naturally occurring FA oligomers but also adds to the understanding of the oligomerization mechanisms in plants. Without accurate quantitation the ability to understand this process via model reactions would be severely hampered.

Quantification of HCAs themselves as small phenolic compounds, which are commercially available in high purities for reasonable prices, does not present many analytical challenges. The compounds need to be extracted from the plant material and separated from co-extracted components before analysis using both chromatographic and non-chromatographic methods. More substantial challenges arise with the quantification of dFAs, tFAs, and higher oligomers of FAs. These compounds are less abundant than the HCAs and are present at levels which may not be detected if the analytical methodology is not specifically designed to detect these compounds such as in screening methods. Methods with adequate sensitivity to detect these compounds still need to be accurate, precise and rugged. The presence of many other compounds in the extracted plant materials is a challenge in developing an accurate, precise, and rugged methodology.

Chromatographic and non-chromatographic methods exist for the quantification of HCAs and ferulate oligomers. Non-chromatographic methods include electrophoresis and spectrophotometry. Chromatographic methods include thin layer chromatography (TLC),

high performance liquid chromatography (HPLC), gas chromatography (GC), and miceller electrokinetic chromatography. Liquid chromatography techniques can be coupled to ultraviolet (UV), photo diode array (DAD), or mass spectrometry (MS) detector to detect FA. GC techniques using both MS or flame ionization (FID) detectors have been used. Other types of spectroscopy have also been employed for the quantification of FA including Fourier Transform Infrared (FTIR) and Fourier Transform-Raman spectroscopy (FT-RS).

2.3 Extraction of Cell-wall Bound Hydroxycinnamic Acids and Ferulic Acid Oligomers and Clean-up Procedures

Ferulates are attached to plant cell wall polysaccharides, lignin and even proteins via ester and ether linkages. These linkages can be cleaved using alkaline or acid hydrolysis in order to free the compounds from their positions in the cell wall, with alkaline hydrolysis being more efficient. However, care must be taken to liberate the compounds without or with minimal alterations of their chemical structures. Alkaline hydrolysis has been shown to result in several modifications of the analytes. Oxidative degradation of the side-chain leading to the formation of aldehydes, e.g. vanillin formation from FA, can be effectively suppressed by nitrogen purging of the sodium hydroxide (NaOH) solution and the headspace of the hydrolysis vessel. The formation of conformational isomers of dFAs, especially of 8-5-coupled dFAs, cannot be suppressed easily, so quantitation must take this into account [1].

After hydrolysis, the liberated HCAs, dFAs and tFAs need to be separated from polysaccharides, proteins, lignins and other components of plant materials. This first extraction is completed using liquid/liquid extraction, solid/liquid extraction or

supercritical fluid extraction [27]. The relative amounts of each group (HCAs, dFAs, tFAs and higher oligomers) can vary widely between plant materials. Primarily there is a large amount of HCAs including monomeric FA, CA and SA in both their *trans*- and, although less abundant, their *cis*-configurations. This dominance of HCAs can make quantification of the dFAs, tFAs and higher oligomers difficult. One potential way to avoid these problems is another pre-separation step. Separation of the extracted compounds based on molecular weight using size exclusion chromatography (SEC) can be used in order to remove the dominating monomers and focus on the dFAs, tFAs and higher oligomers. Once the interfering compounds are removed the extracts can be analyzed.

2.4 Methods to Quantify Ferulic Acid

FA has been quantified using many of the techniques mentioned above. As a small phenolic compound it may be quantified with relative ease. Electrophoresis and micellar electrokinetic chromatography can be used for this purpose but these techniques require several buffers with a relatively small sample capacity and some additional clean-up steps before quantification. Also, these methods show lower accuracy and reproducibility as compared to, for example, HPLC [27]. GC was originally challenging due to the non-volatile nature of FA. However, simple derivatization protocols were developed allowing analysis by GC with excellent selectivity and sensitivity. Among the liquid chromatography techniques, TLC is a relatively simple technique for FA quantification. Although it can be used for quantification it is more commonly used as a preparation and clean-up step. HPLC is the preferred method of quantitation due to its sample capacity, accuracy, sensitivity and efficiency, especially with reversed phase columns.

2.5 Methods to Quantify Ferulic Acid Oligomers

Quantification of higher oligomers of FA has evolved with the identification of these compounds in plant materials. The earliest identified dimer was 5-5–dFA found in wheat germ hemicelluloses and predicted to be a cross-linking compound in plant cell walls [28]. Analysis of dFAs became much more complicated after 1994 when several dFA regioisomers were identified [29] largely increasing the number of analytes and the need for standards for these dFAs to allow for routine identification and quantification. Quantification of dFAs by GC involves the derivitization of the extracted phenolic acids prior to analysis through either methylation or silylation. Detection of the separated, derivatized dFAs is possible using an FID. Identification is assessed through retention time comparison to derivatized standards [30-34]. GC-MS methods have also been developed which provide mass spectral information and increase the ability to identify dFAs [35-37]. Mass spectra for potential dFAs are often compared with synthesized dFAs [34, 36]. Quantification of dFAs has focused on the 5-5-dFA and some photochemically induced cyclodimers prior to 1994. Ralph et al. synthesized several dFA regiosiomers through oxidative coupling reactions and determined response factors for each dFA against *o*-coumaric acid (trimethylsilyl derivatives) [29]. Subsequent GC-FID and GC-MS analysis of dFAs was accomplished using standard compounds synthesized according to this published procedure or retention times and response factors generated using this study as references [38-40]. GC-FID and GC-MS methods are sensitive and very selective and therefore require very little sample preparation or pre-separation of compounds beyond extraction from the plant material and derivatization. However, the stability of the derivatized compounds is an issue as they begin to degrade less than 12

hours after preparation [29]. GC or GC-MS analysis of tFAs or higher oligomers has not been performed yet. This may be due to the large molecular mass of silylated ferulic acid oligomers which in many cases may exceed the mass range of the instrument.

HPLC methods have been developed to separate and quantify dFAs and tFAs. Detection of the compounds is primarily by UV detection with confirmation of the identity of the dFAs or higher oligomers by UV spectra using a DAD. The second detection mode which offers the possibility of confirming the identity of the analytes is MS detection. Ferulate oligomers are often quantified using a single UV wavelength (usually 325 or 280 nm). Compounds of interest are quantified in comparison to internal standards. To do this, synthetic standard compounds [29, 41] or standard compounds isolated from plant materials such as corn bran [42] are necessary to determine response factors or, ideally, correction factors (CF). Selection of the internal standard is critical to accurately quantifying ferulate oligomers. HCAs not normally found in plant materials are often used. However, it would be best to use a compound with a more similar molecular weight and structural similarity to the dFAs and higher oligomers. A fully methylated 5-5-dFA has been synthesized and utilized in a quantitative HPLC method [43]. Most methods use, however, either *o*-coumaric acid or *trans*-cinnamic acid which are not well suited to mimic dFAs [44].

Different from the GC approach, dFAs and higher oligomers do not need to be derivatized for the HPLC approach. However, problems can arise from re-dissolution of the dried extracts for HPLC analysis since the analytes must be completely dissolved in order to provide accurate quantitation. In addition to the quantification of dFAs and tFAs by UV detection, MS approaches have been used for quantitating. Depending on the

starting material some extracts undergo additional clean-up steps and pre-separation using other forms of liquid chromatography (see also chapter 2.3). This, however, is most often used in preparative approaches and rarely in analytical approaches [42, 43, 45-48]. This pre-separation reduces interference from other UV-active, potentially co-eluting compounds. Pre-separation and other clean-up steps may improve the separation of phenolic acids but can be a lengthy process. MS allows dFAs and higher oligomers to be differentiated from each other and other co-extracted plant materials even when chromatographic separation has not been obtained. In addition, this approach can enhance sensitivity of the quantitative method. MS approaches include various ionization sources such as atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) and methods of detection including quadrupole, time-of-flight (TOF), or ion trap. Ferulate oligomers have been monitored in both the positive and negative ionization mode mainly to hypothesize structure and confirm molecular weight [48, 49]. However, quantitative methods have not yet been developed.

Thin layer chromatography (TLC) has also been used as a tool for preliminary identification of higher oligomers and in methods utilizing radioactive labeled compounds to investigate ferulate cross-linking [28, 50]. Though relatively inexpensive and less labor intensive than either GC or HPLC, this method does not have the ability to separate and detect individual ferulate oligomers.

2.6 Overview of Pitfalls Limiting Accurate Quantitation of Hydroxycinnamic Acids and Ferulic Acid Oligomers

While the pros and cons of different methods, that have been used to quantify HCAs, their dimers and higher oligomers, were already mentioned, this section will elaborate on

methodological problems. The chromatographic separation of HCAs from dFAs, tFAs and higher oligomers can be difficult using HPLC methodologies. Large amounts of HCAs can co-elute with the less abundant dFAs, tFAs and oligomers. This problem is further complicated in that many regioisomers of the dimers and higher oligomers are not accounted for. Diferulates with 8-8 and 8-5-linkages can be found in up to three forms each. While the different 8-8-dimers are possibly naturally occurring compounds, alkaline hydrolysis prior to extraction is the driving force behind the formation of the various 8-5-dimers [1]. If the chromatographic method does not account for all of the potential isomers it will underestimate the total dFA content. Most challenging, HCAs, dFAs, tFAs and oligomers occur in varying abundances in plant materials which can complicate peak identification and purity assessment. It is relatively simple to determine if two compounds are partially or completely co-eluting if the compounds are similar in abundance by examining the UV spectra. However, if one of the compounds is much lower in abundance than the compound it is co-eluting with, it may be completely obscured and not visible in the UV spectra. Complicating this issue further, some of the dFAs and tFAs have very similar UV spectra (**Figure 4**).

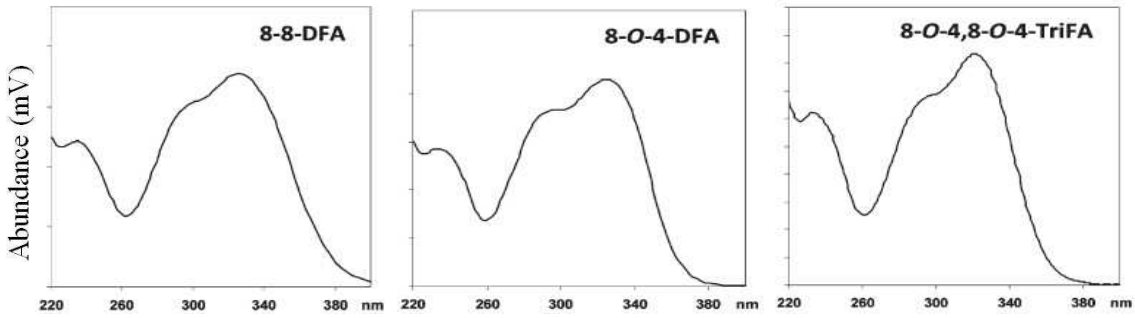

Figure 4. Ultraviolet (UV) spectra for 8-8 non-cyclic dehydrodiferulic acid (8-8-dFA), 8-O-4-dehydrodiferulic acid (8-O-4-dFA) and 8-O-4/8-O-4 dehydrotriferulic acid (8-O-4/8-O4-tFA) [43].

Lastly, HPLC solvent gradients do not fully provide separation of the compounds based on degree of polymerization. This is due to the structural similarities between some of the tFAs and dFAs and can cause problems with identification if the assumption is made that all of the dFAs elute before the tFAs. Some of the problems associated with separation of this diverse class of compounds can be alleviated by pre-separation using size exclusion or the development of individual gradients for the separation of HCAs from the dFAs and higher oligomers [42, 43].

Variation in HPLC instrumentation also contributes to inaccuracies. As mentioned above, several of the dFAs and tFAs have similar UV spectra to monomeric HCAs and each other complicating identification if UV spectra, monitored by a DAD, and retention time are used as parameters for identification. Even less specific is the detection by using a single wavelength UV-detector instead of a DAD. This not only limits the ability to check compound identity and peak purity but may also lead to underestimation of dFAs. The most often used wavelength is 280 nm, some methods, however, use 325 nm or 320 nm. The majority of the compounds absorb UV light at 325 nm, but 8-8THF-dFA d absorbs at neither 325 nm nor 320 nm [43] . Therefore, quantitation at 325 nm neglects the presence of 8-8THF-dFA. Quantitation at 280 nm, while not being the preferred choice to achieve maximum sensitivity for most dFAs and tFAs, will detect all dFAs and tFAs known to date, including the 8-8THF-dFA. While single wavelength detection at 280 nm is possible, use of a two-wavelength UV detector or preferentially a DAD is a better approach [42, 51-53].

Identification of the analytes is a prerequisite for their quantification. Peak identification is often a problem for the dFAs and certainly a problem for tFAs and higher oligomers. While often relative retention times or elution sequences from previous studies are used as a reference they do not provide enough certainty of the identity of the dimers and higher oligomers since many of them have similar UV spectra and retention times. Usage of synthesized or isolated standard compounds would help in peak identification but unfortunately, most groups avoid these laborious tasks as further detailed below. Alternatively, mass spectral analysis is thought to provide information regarding the molecular weight of the compounds and can therefore easily distinguish between dFAs and tFAs. However, differentiation between dFAs and tFAs within the dimer or trimer group, respectively, is difficult if characteristic fragments besides the (quasi)molecular ion are not formed (which is often the case using a single quadrupole MS). Thus, identification based on relative retention times, UV spectra and molecular weight can still give ambiguous results, highlighting the importance of the availability of standard compounds. HCAs are commercially available; however, dFAs, tFAs and higher oligomers of ferulic acid are not. Synthetic pathways for all known dimers exist [29] while tFAs and higher oligomers were formed using model reactions [54-56]. The synthesis of some of the dimers, e.g. the 8-O-4-dFA, and all higher oligomers is very time consuming and often requires advanced synthetic chemistry skills. Additionally, it has to be considered that synthetic pathways using single electron oxidation systems which generally mimic the conditions in the plant well, but structural changes occurring during the alkaline hydrolysis of the plant materials also need to be taken into account. For example, standard compounds should mimic ring openings and the decarboxylation

processes which can occur during the release of the ester-linked compounds by alkaline hydrolysis. Synthetic compounds which cannot occur in plants and which are not formed from naturally occurring compounds during alkaline hydrolysis can be very useful as internal standards, since they very closely mimic the analytes of interest but cannot have their origin from the plant materials. For example, fully methylated 5-5-dFA has been effective as an internal standard but it should be purified before use in order to eliminate any contamination from not fully methylated forms [42, 43]. Isolation of the standard compounds from plant materials provides standard compounds which are identical to the compounds of interest for quantitation in plant materials. dFAs and especially tFAs and higher oligomers occur in very small amounts in plant material and isolation of these compounds is a labor intensive and somewhat difficult process which requires advanced skills in preparative chromatography. Much like the analysis of these compounds in plant materials, alkaline hydrolysis from a source material must first occur. This requires the hydrolysis of large amounts of, for example, insoluble bran fiber from a source that may be pre-screened and determined to contain a relatively high amount of the compounds of interest [42]. After hydrolysis these compounds must be separated using several chromatographic methods including SEC, Sephadex LH-20 chromatography and HPLC, and purified. Identity and purity should be confirmed by nuclear magnetic resonance (NMR).

Even if compounds are unambiguously identified as dFAs or tFAs, their quantification is still challenging. Ferulate oligomers have been quantified based on the usage of an internal standard. Ideally, the internal standard fully accounts for losses during liquid/liquid extraction or other variable steps in the extraction and clean-up procedures.

The usage of an internal standard requires the determination of response and CFs for the compounds analyzed [43]. Many current methods assume that the compounds have the same response to the detector as the internal standard, an assumption which is certainly incorrect. Alternatively, response factors, which were previously reported in literature, are used. While this approach can be useful when exactly the same conditions are applied, differences in the experimental set-up can result in incorrect results. Also, the differentiation between response and CFs needs to be highlighted. While response factors only address the different responses of analytes and the internal standard to the detection method used, corrections factors consider their potentially different behavior during extraction and clean-up, resulting in different recoveries, too.

In order to assess limitations of a method, a defined method validation protocol should be used to evaluate its ability to accurately and precisely analyze the compounds of interest. Besides factors such as selectivity (represented by baseline separation of the analytes in chromatographic approaches), and determination of the linear range (linear relationship between detector signal and analyte concentration), the basic calibration of the method allows the analyst to assess both the sensitivity and the response of the analytes in comparison to the internal standard. Matrix calibration, which approximates the treatment of the sample during extraction, clean-up and analysis, can determine the accuracy of the method through quantifying the recovery rate of each compound. The precision can also be evaluated by determining variation coefficients of the procedure during matrix calibration. Without validation it is impossible to determine how effective the method is at recovering the compounds from the test material and how precisely the compounds are

quantified. The uncertainty generated by non-validated methodologies makes comparison of ferulate oligomers contents between investigations difficult.

A recent validation of a HPLC-UV methodology used to analyze HCAs, dFAs and three tFAs determined, among other factors, accuracy, precision, and sensitivity [43]. The method validation included basic and matrix calibration but only the basic calibration demonstrated an acceptable level of precision. The matrix calibration illuminated the interference caused by other materials in the plant samples such as lignin and the limitations of some clean-up steps resulting in larger variation coefficients if compared to the basic calibration and more importantly poor accuracy (low recovery rates) for several compounds tested. This method validation was the first publication to include validation data in the development of a methodology to determine dFAs and tFAs and discuss the limitations inherent to this approach. One such limitation resulting in poor accuracy is the extraction of the compounds from the hydrolyzed plant material using liquid to liquid extraction. Another potentially severe limitation is the re-dissolution of the extracted compounds into a solvent suitable for HPLC analysis. Finally, degradation products, of lignin in particular, were found to cause co-elution problems that could not be resolved with UV detection. Dobberstein and Bunzel suggested that the extraction and re-dissolution issues should be investigated further to potentially resolve some of the problems of low recovery rates [43]. Use of MS detection was suggested as a solution for problems resulting from the co-elution of lignin degradation products with analytes. Most importantly, this method validation demonstrated the need to adjust quantification data by using CFs in order to more accurately quantify dFAs and tFAs.

2.7 Current Quantitative Data on Ferulic Acid Oligomers in Plant Materials

Dimers and some higher oligomers of FA have been quantified using the methods described in Chapter 2.5. Data on dFA contents are summarized in (**Table 1)**. dFAs which have been identified and quantified in plant material are shown in (**Figure 5**). Higher oligomers have not been quantified as commonly as dFAs. The concentrations of 5-5/8-O-4-tFA, 8-8c/8-O-4-tFA and 8-O-4/8-O-4-tFA (**Figure 6**) in maize grain insoluble fibers were determined to be 788, 55 and 73 µg/g [43]. Other studies only focused on the analysis of 5-5/8-O-4-tFA, most often in wheat and its different milling fractions and grain layers [57]. Other tFAs, which were identified in certain plant materials (**Figure 6**), have yet to be quantified.

Table 1. Diferulate contents (dehydrodiferulic acids and/or cyclobutane dimers) of plant materials

Year	Study authors	Plant	Tissue	Total diferulates [µg/g]	Diferulates quantified[a]
1990	Eraso and Hartley	Ryegrass	Isolated cell walls	280	cyclobutane dimers
		Barley straw	Isolated cell walls	450	cyclobutane dimers
		Lucerne stem	Isolated cell walls	100	cyclobutane dimers
		Red clover stem	Isolated cell walls	100	cyclobutane dimers
1990	Ford and Hartley	Pangola Stem	Isolated cell walls	2460	cyclobutane dimers
		Setaria Steam	Isolated cell walls	880	cyclobutane dimers
		Pangola Leaf	Isolated cell walls	640	cyclobutane dimers
		Setaria Leaf	Isolated cell walls	1410	cyclobutane dimers
		Spear grass	Isolated cell walls	370	cyclobutane dimers
1990	Hartley et al.	Coastal Bermuda grass	Isolated cell walls	3890	cyclobutane dimers
1991	Hartley and Morrison	Tall fescue	Isolated cell walls	2210	cyclobutane dimers
		Bermuda grass	Isolated cell walls	3250	cyclobutane dimers
1994	Ralph et al.	Cocksfoot	Isolated cell walls	11610	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Switchgrass	Isolated cell walls	19000	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
1996	Parr et al.	Chinese water chestnut	Isolated cell walls	12221	8-O-4,8-5c,5-5
1996	Waldron et al.	Wheat straw	Isolated cell walls	475	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5
1997	Bartolome et al.	Spent barley	Isolated cell walls	2372	8-8c,8-5,8-O-4,8-5c,5-5
		Wheat	Isolated cell walls	3127	8-8c,8-5,8-O-4,8-5c,5-5
1998	Garcia-Conesa et al.	Wheat	Isolated cell walls	1303	8-O-4,8-5c,5-5
1998	Figueroa-Espinoza et al.	Wheat	Isolated WEAX[b]	4044 to 4920	8-8c,8-5,8-O-4,8-5c,5-5
1998	Lempereur et al.	Wheat	Middlings	677.7	8-5nc,8-O-4,8-5c,5-5
			Wheat flour	297.7	8-5nc,8-O-4,8-5c,5-5
1999	Saulnier et al.	Maize	Bran	13300	8-5nc,8-O-4,8-5c,5-5

Table 1. (continued)

Year	Study authors	Plant	Tissue	Total diferulates [µg/g]	Diferulates quantified[a]
2000	Renger and Steinhart	Barley	Dietary fiber/bran	744	8-8c,8-8,8-5,8-O-4,5-5
		Maize	Dietary fiber/bran	1016	8-8c,8-8,8-5,8-O-4,5-5
		Oats	Dietary fiber/bran	466	8-8c,8-8,8-5,8-O-4,5-5
		Rye	Dietary fiber/bran	713	8-8c,8-8,8-5,8-O-4,5-5
		Wheat	Dietary fiber/bran	808	8-8c,8-8,8-5,8-O-4,5-5
2000	Andreasen et al.	Rye	Whole grain	241 to 409	8-5nc,8-O-4,8-5c,5-5
2000	Andreasen et al.	Rye	Bran >0.71mm	980	8-5nc,8-O-4,8-5c,5-5
		Rye	Bran <0.71 mm	686	8-5nc,8-O-4,8-5c,5-5
		Rye	Flour	82	8-5nc,8-O-4,8-5c,5-5
		Rye	Whole grain	307	8-5nc,8-O-4,8-5c,5-5
2001	Bunzel et al.	Maize	Whole grain	12655	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Wheat	Whole grain	2556	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Barley	Whole grain	3727	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Rye	Whole grain	3730	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Oat	Whole grain	3637	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Spelt	Whole grain	2834	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Rice	Whole grain	4042	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Wild Rice	Whole grain	2840	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc
		Millet	Whole grain	5739	8-8c,8-8nc,8-5nc,8-O-4,8-5c,5-5,4-O-5,8-5dc

Table 1. (continued)

Year	Study authors	Plant	Tissue	Total diferulates [µg/g]	Diferulates quantified[a]
2006	Gallardo et al.	Wheat	Middlings	224	8-5,8-O-4,8-5c,5-5
			Separated middlings	130	8-5,8-O-4,8-5c,5-5
			Flour	80	8-5,8-O-4,8-5c,5-5
			Germ	133	8-5,8-O-4,8-5c,5-5
			Bran	287	8-5,8-O-4,8-5c,5-5
			Fermented bran	399	8-5,8-O-4,8-5c,5-5
			Cold pressed germ	72	8-5,8-O-4,8-5c,5-5
		Rye	Flour	37	8-5,8-O-4,8-5c,5-5
			Middlings	201	8-5,8-O-4,8-5c,5-5
			Bran	225	8-5,8-O-4,8-5c,5-5
		Buckwheat	Husks	0	8-5,8-O-4,8-5c,5-5
			Flour	0	8-5,8-O-4,8-5c,5-5
2010	Dobberstein and Bunzel	Maize	Bran	13965	8-8c,8-8THF,8-8nc,8-5nc,8-O-4,8-5c,5-5,8-5dc
		Wheat	Whole grain	1956	8-8c,8-8THF,8-8nc,8-5nc,8-O-4,8-5c,5-5,8-5dc
		Rye grain	Whole grain	1925	8-8c,8-8THF,8-8nc,8-5nc,8-O-4,8-5c,5-5,8-5dc
		Sugar beet	Whole grain	1586	8-8c,8-8THF,8-8nc,8-5nc,8-O-4,8-5c,5-5,8-5dc
		Asparagus	Whole grain	545	8-8c,8-8THF,8-8nc,8-5nc,8-O-4,8-5c,5-5,8-5dc
		Maize	Stover	2091	8-8c,8-8THF,8-8nc,8-5nc,8-O-4,8-5c,5-5,8-5dc
2011	Lygin et al.	Switchgrass	Stem	700	8-8c,8-5,8-O-4,5-5
		Miscanthus	Stem	750 to 1450	8-8c,8-5,8-O-4,5-5
		Reed arundo	Stem	500	8-8c,8-5,8-O-4,5-5

[a] c – cyclic, dc – decarboxylated, nc – noncyclic, THF – tetrahydrofuran, [b]WEAX – water extractable arabinoxyla

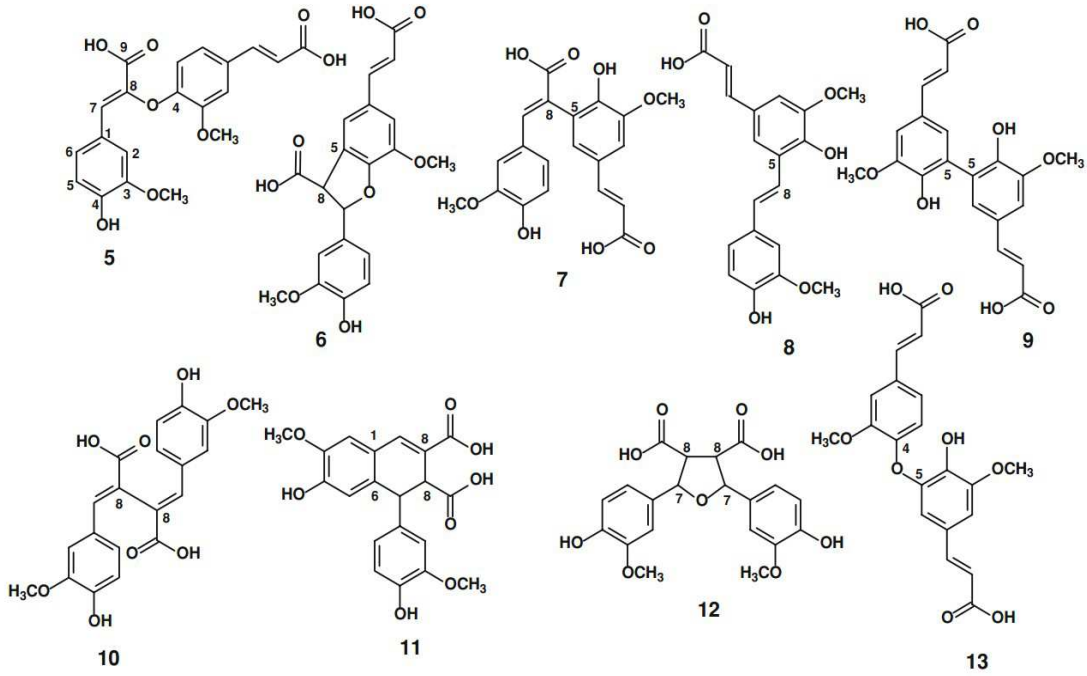

Figure 5. Dehydrodiferulic acids (dFAs) identified in different plant materials. The cyclic form is abbreviated with c, the open (non-cyclic) form with nc, the decarboxylated form with dc, and THF indicates a tetrahydrofuran substructure. 5, 8-O-4-dFA; 6, 8-5c-dFA; 7, 8-5nc-dFA; 8, 8-5dc-dFA; 9, 5-5-dFA; 10, 8-8nc-dFA; 11, 8-8c-dFA; 12, 8-8THF-dFA; 13, 4-O-5-dFA [1].

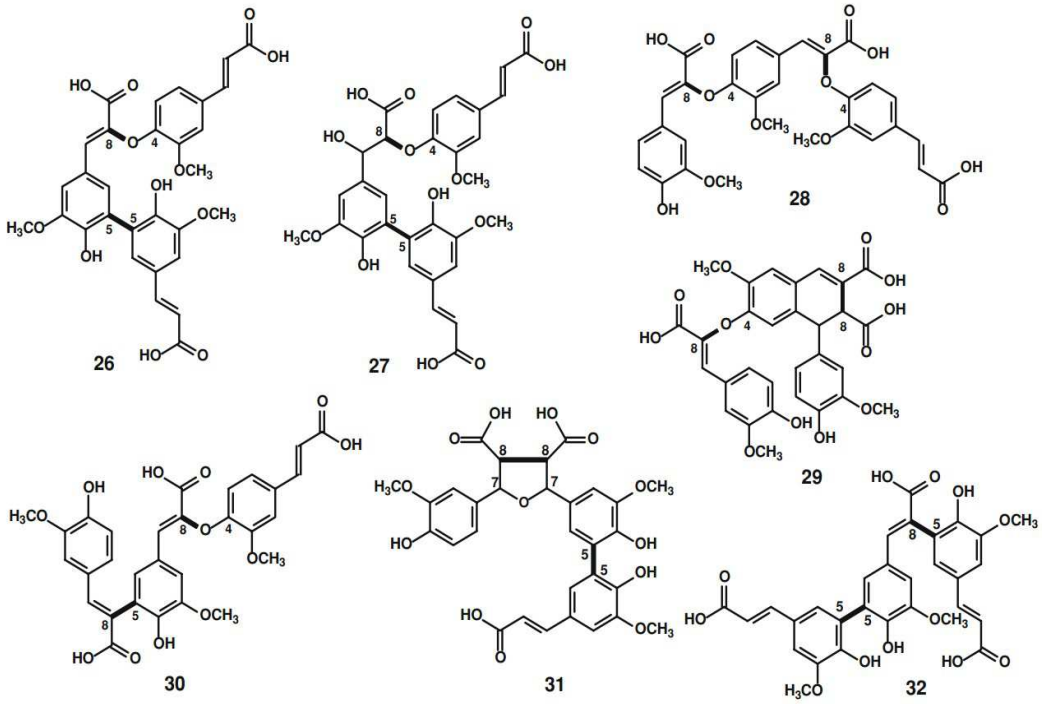

Figure 6. Dehydrotriferulic acids (tFAs) isolated from maize bran after alkaline hydrolysis. 26, 5-5/8-O-4-tFA; 27, 5-5/8-O-4(H_2O)-tFA; 28, 8-O-4/8-O-4-tFA; 29, 8-8c/8-O-4-tFA; 30, 8-O-4/8-5nc-tFA; 31, 8-8THF/5-5-tFA; 32, 8-5nc/5-5-tFA. c, cyclic form; nc, open (non-cyclic) form; THF, tetrahydrofuran form [1].

Chapter 3 Manuscript

3.1 Introduction

HCAs, a sub-class of phenolic acids found in plants, can participate in photochemically or oxidatively induced coupling. As such, in plant materials they have been found to oxidatively couple with plant cell wall components such as polysaccharides, lignin, proteins, and each other forming homo or heterodimers via ester and ether linkages [1]. This coupling plays an important role in the plant cell wall chemistry contributing to cell wall strength and influencing the physiochemical properties of plant materials. FA in particular has been found to oxidatively couple to produce dimers, trimers and higher oligomers of FA ester linked to plant cell wall components [1]. Alkaline hydrolysis of these ester linkages releases the bound HCAs, dFAs and tFAs which have previously been quantified using primarily GC and HPLC methodologies [27]. GC analysis allows HCAs and dFAs to be completely separated during analysis but they must be derivitized prior to analysis. Derivitized ferulate oligomers larger than dFAs may be too large to analyze via traditional GC methodologies. HPLC methodologies do not have similar size limitations and do not require derivitization prior to analysis. However, HPLC methodologies currently in use do not have the ability to completely separate the more abundant HCAs from the dFAs or dFAs from tFAs. This leads to coelution issues which cannot be easily resolved with the use of UV detection. These coelution issues lead to the underestimation of the 8-8-dFAs in particular as coelution with the more dominant HCAs can obscure their presence [1]. Most methods have not been validated due to difficulty

associated with obtaining appropriate standard compounds. A recent validation of a HPLC-UV methodology developed using a two gradient approach eliminated the coelution of HCAs with dFAs and tFAs [43]. However, co-elution with other compounds present in hydrolyzed plant materials, such as lignin degradation products, highlights the difficulty associated with using UV as the sole detection source [43]. The aim of this paper is to describe the development, application and validation of a methodology using MS detection for the quantification of dFAs and tFAs in plant materials.

3.2 Materials and Methods

Materials and Instrumentation. 5-5 Methylated(Me)-dFA which was used as an internal standard was kindly donated by Prof. John Ralph (Department of Biochemistry, University of Wisconsin, Madison, WI). Cellulose was obtained from Sigma (St. Louis, MO), xylan (from oat spelts) was from Tokyo Chemical Industry Co., LTD. (Tokyo, Japan). Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.4 L (EC 3.4.21.62, from *B. licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) from Novozymes (Franklinton, NC). Sephadex LH-20 was from GE Healthcare Biosciences (Pittsburgh, PA).

The SEC column was purchased from TOSOH Bioscience LLC (King of Prussia, PA), semi-preparative and analytical phenyl-hexyl HPLC columns were from Phenomenex (Torrance, CA). Solvents [tetrahydrofuran (THF), methanol (MeOH) and acetonitrile (ACN)] were HPLC grade, water was distilled and deionized. SEC instrumentation L-6200A intelligent pump was from Hitachi (Pleasanton, CA), the UV/VIS Spectra 200

programmable wavelength detector was from Spectra Physics (Irvine, CA) and the 6125 six way injection valve was from Rheodyne (Rhonert Park, CA). Sephadex LH-20 chromatography instrumentation also utilized the Spectra 200 detector with an ISO-100 isocratic digital pump from Chrom Tech (Apple Valley, MN). Semi-preparative and analytical HPLC instrumentation for isolation and identification of standard compounds was comprised of LC-20AT solvent delivery system, CBM-20A communication bus module, CTO-20A column oven, SPD-20A UV/VIS photodiode array (PDA) detector and SIL-10AF autosampler for analytical HPLC from Shimadzu (Columbia, MD) or an 7725 six channel injection valve Rheodyne (Rhonert Park, CA) for semi-preparative HPLC. A binary pump LC-10AD vp, flow controller FCV-10AL vp, column oven CTO-10A vp, system controller SCL-10A vp, and UV detector SPD-6AV used to perform HPLC-MS were from Shimadzu (Columbia, MD). The Micromass Quattro MS was from Waters (Milford, MA). NMR experiments were performed on a Bruker 700 MHz Avance NMR Spectrometer (Rheinstetten, Germany).

Cereral and Pseudocereal Flours. Maize bran was provided by Cargill (Indianapolis, IN). Whole grain barley flour (stone ground), whole grain oat flour, whole grain maize flour (stone ground), organic whole grain buckwheat flour, organic whole grain amaranth flour (stone ground), organic whole wheat flour (stone ground) and yellow popcorn were obtain from Bob's Red Mill Natural Foods (Milwaukie, OR). Whole yellow popcorn and maize bran were ground to a particle size of < 0.5 mm particle size using a Brinkmann ZM-1 centrifugal mill from Retsch (Haan, Germany) prior to analysis.

Isolation of Standard Compounds. With the exception of 4-O-5-dehydrodiferulic acid all known dFAs and five major tFAs [8-5non-cyclic (nc)/8-O-4-, 8-5nc/5-5-, 8-8cyclic (c)/8-O-4-, 5-5/8-O-4-, 8-O-4/8-O-4-tFA] were isolated from the alkaline hydrolysate from maize bran using different chromatographic separations as described below.

Preparation of Insoluble Maize Fiber. Milled maize bran (approximately 400 g) was defatted by three consecutive acetone extractions (1.0 L of solvent each, 1 h with shaking per extraction). An enzymatic procedure (heat-stable α-amylase, protease and amyloglucosidase) used previously [42] was scaled up to prepare the insoluble corn fiber. In the scaled up procedure, approximately 400 g of maize bran suspended in 1.3 L of phosphate buffer were processed with α-amylase (8.8 mL), protease (8.8 mL) and amyloglucosidase (10.3 mL).

Alkaline Hydrolysis and Extraction. Saponification of insoluble maize fiber (220 g) and extraction of phenolic acids was performed as previously described (Bunzel, Funk and Steinhart, 2004 J Sep Sci) with the following modifications: hydrolysis was performed in 500 mL centrifuge bottles and liberated phenolic acids were extracted into ethyl acetate. Materials were upscaled to: 4 x 10 g of bran, 200 mL of 2 M NaOH, ~40 mL of 37% HCL (acidification prior to ethyl acetate extraction), 150 mL of ethyl acetate for the first extraction followed by 2 x 80 mL ethyl acetate extractions. Pooled ethyl acetate extracts were reduced to 100 mL, 4 x 50 mL of 5% $NaHCO_3$ were used for clean-up, and 3 x 80 mL of ethyl acetate were used to re-extract the phenolic acids from the acidified $NaHCO_3$ solution.

Size Exclusion Chromatography. Dried maize fiber hydrolysates were redissolved in THF (120 mg/mL) and separated according to their molecular weight by SEC. Dissolved hydrolysates were injected (475 µL) onto a TSK Gel G1000H column (30 cm, 21.5 mm i.d., 5 µm) using a 500 µL sample loop. Separation was performed isocratically at 25 °C using a flow rate of 4.0 mL/min. Chromatograms were monitored at 325 nm using a preparative scale flow cell. The following fractions were collected by hand, according to the chromatogram: monomeric, dimeric and oligomeric (trimeric and higher) phenolic acids. Fractions were pooled, evaporated to dryness under reduced pressure and further dried under high vacuum for at least 8 h.

Sephadex LH-20 Chromatography. The pooled SEC fractions which contained dFAs were further separated using Sephadex-LH-20 chromatography according to previous conditions [42] with minor modifications. Fractions were re-dissolved in 0.5 mM aqueous trifluoroacetic acid (TFA)/MeOH 50/50 (v/v) to an approximate concentration of 50 mg/mL. Approximately 500 mg of sample were applied to the pre-conditioned (0.5 mM aqueous TFA/MeOH 95/5 (v/v)) column (gel bed: 60 cm x 2.5 cm) and elution was performed in four steps:

1) elution with 0.5 mM aqueous TFA/MeOH 95/5 (v/v) for 48 h, flow rate 1.5 mL/min;

2) elution with 0.5 mM aqueous TFA/MeOH 50/50 (v/v) for 43 h, flow rate 1.0 mL/min;

3) elution with 0.5 mM aqueous TFA/MeOH 40/60 (v/v) for 60 h , flow rate 1.0 mL/min;

4) rinsing step with 100% MeOH.

A UV-detector with a preparative flow cell was used to monitor the effluent at 280 nm. Fractions were collected every 18 min and combined according to the chromatograms.

Dried fractions were further separated/purified by semi-preparative RP-HPLC as described below. SEC fractions expected to contain tFAs were redissolved as described for the dFA fraction and injected onto the column. After a 5 min elution with MeOH to rinse the material out of the injection loop, the elution solvent was switched to 0.5 mM aqueous TFA/MeOH 80/20 (v/v) (flow rate 1.0 mL/min). The 5 min elution with MeOH was necessary to avoid early precipitation of the sample. Elution and fractionation were carried out as described for the dFA fractions. A total of approximately 2.6 g of dFAs and 1.0 g of tFAs were separated as described.

Further Fractionation or Purification of Dehydrodiferulic and Dehydrotriferulic Acids. Further separation and purification of Sephadex LH-20 fractions was performed by semi-preparative RP-HPLC on a Luna phenyl-hexyl column (250 mm x 10 mm i.d., 5 µm particle size) and a ternary gradient system made up of 1 mM aqueous TFA (eluent A), ACN (eluent B) and MeOH (eluent C) at a flow rate of 2.5 mL/min. The injection volume was 500 µL and separations were performed at either 35°C or 45°C. Chromatograms were monitored at 280 nm and 325 nm. Dried Sephadex LH-20 fractions were redissolved in either water/MeOH 50/50 (v/v) (most dFAs) or water/MeOH/acetone 50/25/25 (v/v/v) (tFA*s) in order to fully dissolve material and prevent subsequent precipitation. The following gradients were used for further separation and purification of dFAs and tFAs:

- 8-8nc-dFA, 8-8c-dFA and 8-8tetrahydrofuran(THF) form-dFA: initially 87% A/13% C, linear over 15 min to 84% A/16% C, linear over 5 min to 82% A/18% C, linear over 5 min to 75% A/3% B/22% C, linear over 5 min to 73% A/5% B/22% C, linear over 10

min to 68% A/5% B/27% C, held for 5 min at 68% A/5% B/27% C, linear over 10 min to 59% A/5% B/36% C, linear over 5 min to 19% A/36% B/45% C, equilibrated back to initial conditions and held for 10 min.

- 8-5nc-dFA and 5-5-dFA: initially 87% A/13% C, linear over 5 min to 75% A/3% B/22% C, linear over 10 min to 73% A/5% B/22% C, linear over 25 min to 68% A/5% B/27% C, linear over 5 min to 59% A/5% B/36% C, linear over 5 min to 19% A/36% B/45% C, equilibrated back to initial condition and held for 5 min.
- 8-O-4-dFA and 8-5c-dFA: initially 87% A/13% C, linear over 5 min to 79% A/3% B/18% C, linear over 5 min to 73% A/5% B/22% C, linear over 10 min to 68% A/5% B/27% C, held for 5 min at 68% A/5% B/27% C, linear over 7 min to 50% A/5% B/45% C, equilibrated back to initial conditions and held for 5 min.
- 8-5decarboxylated(dc)-dFA: initially 87% A/13% C, linear over 15 min to 84% A/16% C, linear over 5 min to 82% A/18% C, linear over 5 min to 75% A/3% B/22% C, linear over 5 min to 73% A/5% B/22% C, linear over 10 min to 68% A/5% B/27% C, held for 5 min at 68% A/5% B/27% C, linear over 7 min to 50% A/5% B/45% C, equilibrated back to initial conditions and held for 5 min.
- 8-5nc/8-O-4-tFA: initially 87% A/13% C, linear over 2 min to 84% A/16% C, linear over 3 min to 82% A/18% C, linear over 3 min to 75% A/3% B/22% C, linear over 5 min to 73% A/5% B/22% C, linear over 10 min to 68% A/5% B/27% C, held for 5 min at 68% A/5% B/27% C, linear over 10 min to 59% A/5% B/36% C, linear over 5 min to 19% A/36% B/45% C, equilibrated back to initial conditions and held for 5 min.
- 8-5nc/5-5 tFA: initially 87% A/13% C, linear over 8 min to 73% A/5% B/22% C, linear over 10 min to 68% A/5% B/27% C, linear over 9 min to 59% A/5% B/36% C, linear

over 6 min to 19% A/36% B/45% C, equilibrated back to initial conditions and held for 5 min.

- 8-8c/8-O-4-tFA and 5-5/8-O-4-tFA: initially 87% A/13% C, linear over 8 min to 73% A/5% B/22% C, linear over 10 min to 68% A/5% B/27% C, linear over 17 min to 59% A/5% B/36% C, linear over 5 min to 19% A/36% B/45% C, equilibrated back to initial conditions and held for 5 min. 8-O-4/8-O-4-tFA: initially 87% A/13% C, linear over 8 min to 73% A/5% B/22% C, linear over 10 min to 68% A/5% B/27% C, linear over 27 min to 59% A/5% B/36% C, linear over 5 min to 19% A/36% B/45% C, equilibrated back to initial conditions and held for 5 min.

Confirmation of Identity and Determination of Purity of the Isolated Standard Compounds. Identity and purity of the isolated standard compounds were evaluated by using both analytical RP-HPLC-PDA detection and ^{1}H-NMR experiments. Analytical RP-HPLC-PDA was performed using a Luna phenyl-hexyl column (250 mm x 4.6 mm i.d., 5 µm particle size, plus 3 mm x 4.6 mm i.d. guard column) and ternary gradient systems of 1 mM aqueous TFA (eluent A), MeOH (eluent B) and ACN (eluent C). Injection volume was 20 µL, separation was performed at 45°C and chromatograms were monitored at 280 nm. Compounds were separated by using the following gradient: initially 87% A/13% C, linear over 15 min to 84% A/16% C, linear over 5 min to 82% A/18% C, linear over 5 min to 75% A/3% B/22% C, linear over 5 min to 73% A/5% B/22% C, linear over 5 min to 68% A/5% B/27% C, held for 5 min at 68% A/5% B/27% C, linear over 10 min to 59% A/5% B/36% C, linear over 5 min to 19% A/36% B/45% C, equilibrated back to initial conditions and held for 10 min. Purified isolated standard

compounds were dissolved in ethanol-d_6 or acetone-d_6 to asses purity by ^{1}H-NMR. Proton spectra of the purified isolated standard compounds were calibrated using the residual solvent peak. Isolated standard compound spectra were analyzed for signals from impurities. Purity was assessed by examining the integrals of the impurity signals compared to those of the standard compound signals. Isolated standard compounds with estimated impurity signals of less than 5% were retained for standard compound use.

Method Development and Validation. The following dFAs and tFAs were included in the method (in order of elution on the phenyl hexyl column): 8-8c-, 8-8nc-, 8-8THF-, 8-5nc-, 5-5-, 8-O-4-, 8-5c-, 5-5Me-, and 8-5dc-dFA; and 8-5nc/8-O-4-, 8-5nc/5-5-, 8-8c/8-O-4-, 5-5/8-O-4-, 8-O-4/8-O-4-tFA. Basic and matrix calibration were performed in order to determine the accuracy and precision of the method. The tested concentration range for dFAs was 3.0 to 30.0 µg/mL (steps 3.0, 9.8, 16.5, 23.3 and 30.0 µg/mL) and for tFAs 0.5 to 5.0 µg/mL (steps 0.5, 1.6, 2.8, 3.9 and 5.0 µg/mL). The homogeneity of variances across the tested concentration range was determined by measuring the highest and lowest concentrations six times and performing an F-test. Calibration standards for basic and matrix calibrations were measured three times at each concentration level. Appropriate amounts of the standard compounds were dissolved in MeOH/H_2O 50/50 (v/v) and this stock solution was diluted to the required concentrations in dioxane/H_2O 50/50 (v/v) to perform a basic calibration. Each concentration was prepared in triplicate and analyzed using the RP-HPLC-MS methodology described below. Selectivity (baseline separation, less than 10% peak overlap), homogeneity of variances, detection limit (signal to noise ratio of 3:1), quantitation limit (signal to noise ratio of 9:1), linearity

(visual examination and correlation coefficients), sensitivity (slope) and precision (residual standard deviation) were examined for the basic calibration.

A matrix calibration was performed using cellulose and xylan in a 50/50 (w/w) ratio to mimic the plant cell wall matrix. The matrix polysaccharides (75 mg) were saponified as detailed below for destarched cereal and pseudocereal flours and the hydrolysates were spiked with aliquots of stock solutions of the dFAs and tFAs prior to acidification. The added standard compounds were extracted from the matrix with diethyl ether (2 x 2 mL followed by a third 1 mL extraction) which was pooled, evaporated and redissolved in dioxane/H_2O 50/50 (v/v) (500 µL) for HPLC-MS analysis. The matrix calibration was also tested using the same parameters as the basic calibration mentioned above. The standard deviation of the procedure and the coefficient of variation of the procedure were calculated for both the basic and matrix calibrations. The standard deviation of the procedure was calculated by obtaining the quotient of the residual standard deviation and the slope of the regression line. The standard deviation of the procedure was divided by the center of the tested concentration range and multiplied by 100 to calculate the coefficient of variation for the procedure (expressed as a %). Also, the recovery rate was calculated by plotting the spiked concentrations (known concentration added to each matrix calibrant) against the analyzed concentrations (calculated concentration in each matrix calibrant using regression line equations generated from the basic calibration) and determining the slope of the regression line generated. CFs against 5-5Me-dFA as the internal standard for the determination of dFAs and tFAs were calculated from the matrix calibration. CFs were calculated by plotting the concentration (analyte) x area (internal

standard) on the *y*-axis vs. concentration (internal standard) x area (analyte) on the *x*-axis and calculating the slope of the resulting regression line.

A mixture of the isolated standard compounds were analyzed by HPLC-MS using ESI operating in the negative and positive ionization modes with a capillary voltage of 4 kV, cone voltage of 30 V, source temperature of 140°C, desolvation temperature of 415°C, desolvation gas flow of 550 L/h and a gas flow through the cone at 60 L/h. Analysis of the standard mixture covered the mass to charge (m/z) range of 100 to 800 atomic mass units (amu) and was performed in order to identify parent and fragment ions. Mass spectra generated for the standards were examined to select unique and abundant ions for the development of select ion recording (SIR) methods. Standard compounds which were found to coelute were reanalyzed using individual standards and unique ions were identified which can allow for differentiation of the coeluting compounds using SIR. The quasi molecular ions $[M-H]^-$ generated during negative ionization were determined to be the most feasible ions to select for SIR analysis.

Alkaline Hydrolysis of Cereal and Pseudocereal Flours and Extraction of Phenolic Acids. Cereal and pseudocereal flours (1 g) were defatted with hexane (10 mL, 4 h, constant shaking), centrifuged, and dried in an oven at 60°C for one hour followed by drying at 40°C overnight. Defatted flours were suspended in 14.8 mL of distilled water, heat stable α-amylase (Termamyl, 200 µL) was added and flours were incubated for 60 min at 90°C in a heated water bath with occasional shaking. Destarched flours were removed from the heated bath, cooled down to room temperature and 5 mL of 8 M NaOH (degassed with N_2 prior to addition) was added. Hydrolysis was performed overnight

while protected from light. After hydrolysis *o*-hydroxycinnamic acid (o-HCA) (11.8 µL, 0.530 µg/µL) and 5-5Me-dFA (75 µL, 0.2002 µg/µL) were added to the hydrolyzed samples as internal standards. The suspensions were acidified to pH <2 with 37% HCl (5 mL) and extracted three times with diethyl ether (2 x 8 mL, 4 mL). Diethyl ether extracts were pooled, washed with acidified water (5 mL, pH<2) and dried under a stream of nitrogen. The extracts were redissolved in H_2O/dioxane 50/50 (v/v) (500 µL) and analyzed for their dFA and tFA contents by RP-HPLC-MS.

Phenyl-hexyl Reverse Phase-High Performance Liquid Chromatography of Dehydrodiferulic and Dehydrotriferulic acids. Sample extracts were separated and quantified using an HPLC coupled to a UV detector and single quadrupole mass spectrometer. The effluent was split in a 3:1 ratio with 3 parts of the effluent going to the UV-detector (single wavelength at 280 nm) and 1 part going to the MS. Sample extracts were separated using a ternary solvent gradient [aqueous formic acid 0.1% (v/v) (eluent A), MeOH (eluent B), ACN (eluent C)] on a Luna phenyl-hexyl column (250 mm x 4.6 mm i.d., 5 µm particle size, with a 3 mm x 4.6 mm i.d. guard column) at a flow rate of 1.0 mL/min. The sample was injected (40 µL into a 20 µL sample loop) onto the column using a six-port valve and separated using the following gradient:

- Initially 87% A/13% C, increased linearly over 3.5 min to 84% A/16% C, linear over 3.5 min to 82% A/18% C, linear over 10 min to 75% A/3% B/22% C, linear over 10 min to 73% A/5% B/22% C, linear over 15 min to 68% A/5% B/27% C, held for 5 min at 68% A/5% B/27% C, linear over 10 min to 59% A/5% B/36% C, linear over 3 min to 19% A/36% B/45% C and re-equilibrated at initial conditions (87% A/13% C) for 2 min.

Ionization was performed by ESI operating in the negative ionization mode as described above. Analyte peaks were monitored through SIR separated into two channels. Monitored ions for the dFAs were 385 $[M-H]^-$ (8-8c-, 8-8nc-, 8-5nc-, 5-5-, 8-O-4-, 8-5c-dFA)], 341 $[M-H]^-$ (8-5dc-dFA)], 399 $[M-H]^-$ (5-5Me-dFA)] on one channel and 403 $[M-H]^-$ (8-8THF-dFA)] and for the tFAs, 577 $[M-H]^-$ on the second channel$^-$ with a scan dwell time of 1 sec for 403 and 577 and 0.5 sec for 341, 385 and 399. If necessary, sample extracts were diluted in H_2O/dioxane 50/50 (v/v) to stay within the tested concentration range for the analytes.

3.3 Results and Discussion

The determination of dFAs and tFAs is often plagued by problems with specificity and sensitivity to this class of phenolic compounds [43, 44]. Previously, an HPLC-UV method was developed by our group, reducing the separation problems which resulted in the coelution of dFAs with late eluting HCA monomers [43]. The method uses different HPLC gradients for the determination of a) monomeric phenolic acids and aldehydes, and b) dFAs and tFAs. Unfortunately this method while able to alleviate separation issues can still be complicated by the presence of, for example, UV-active lignin degradation products leading to co-elution problems. In addition, many of the compounds have very similar UV spectra which can make the identification of peaks in the event of retention time shifts very difficult. The tFAs have comparably high molecular weights and are thought to be present in plant materials at much lower levels than dFAs [43]. This makes their analysis particularly difficult since higher molecular weight phenolic compounds can be difficult to dissolve leading to sample preparation issues and, due in part to their low abundance, they possibly remain undetected if they coelute with more abundant

compounds such as the dFAs. These issues complicate identification and certainly quantitation of the full set of known dFAs and tFAs in plant materials using a HPLC-UV methodology.

Development of a Gas Chromatography-Mass Spectrometry Method. Previously, GC-MS methodologies have been used to successfully identify and semi-quantify dFAs [29, 40, 58]. Increased resolution observed in GC as compared to HPLC avoids extensive coelution which should facilitate quantification of dFAs by GC. Phenolic acids from plant materials can be derivatized with minimal sample preparation and mass spectral information provides additional structural information about the compounds. However, derivatized dFAs, i.e. trimethylsilylated dFAs, have demonstrated stability issues [29]. tFAs were not previously analyzed with GC-MS. Due to their large molecular weight, which is further increased by trimethylsilylation, it was thought unfeasible to separate and detect them using traditional GC-MS(single quadrupole) techniques using conventional capillary columns. Our group developed a methodology for the separation and detection of dFAs as well as tFAs using high temperature GC-MS. Trimethysilylated dFAs and tFAs were separated using a stainless steel capillary column coated with a standard polysiloxane phase. This allowed the maximum analysis temperature to be extended from 325°C to 430°C and facilitated elution of the higher molecular weight derivatized tFAs. This method also utilized a stepped temperature program (**Table 9**, **Appendix**) to separate the HCA monomers, dFAs and tFAs into three distinct groups as shown in (**Figures 17, 18 and 19**) in the appendix. While separation and detection was

obtained, the methodology was not able to fulfill several validation parameters making it unsuitable for quantitative determination of the dFAs and tFAs in plant materials.

Development of a High Performance Liquid Chromatography-Mass Spectrometry Method. The difficulties in quantification of these compounds highlighted by the limitations of the HPLC/UV and GC-MS methodologies, namely low abundance, less characteristic UV-spectra, coelution, low volatility of the silylated tFAs, and reduced sample stability of the silylated dFAs and tFAs, may be reduced by using an HPLC-MS approach. Thus, an HPLC method combining the selectivity and sensitivity provided by mass spectrometry with the conventional sample preparation for HPLC (eliminating the need of derivatization) was developed for dFA and tFA analysis in cereal grains. dFAs and tFAs were isolated from corn bran alkaline hydrolyzates by consecutive fractionation using SEC, Sephadex LH-20 chromatography, and RP-HPLC. Quantities and purities of the isolated standard compounds used for method development and validation are shown in (**Table 2**).

Table 2. Quantities and purities of the isolated dehydrodiferulic acids (dFA)a and dehydrotriferulic acids (tFA) used for method development and validation

	amount (mg)	purity[b] (%)
8-8c-dFA	21.7	>95
8-8nc-dFA	24.6	>95
8-5nc-dFA	76.9	>95
8-8THF-dFA	34.8	>95
8-O-4-dFA	96.9	>99
8-5c-dFA	5.2	>95
5-5-dFA	184	>99
8-5dc-dFA	12.5	>99
8-8c/8-O-4-tFA	9.0	>97
8-5nc/5-5-tFA	11.3	>97
8-5nc/8-O-4-tFA	3.4	>97
5-5/8-O-4-tFA	11.1	>99
8-O-4/8-O-4-tFA	3.3	>95

[a] c - cyclic form, nc - open (non-cyclic) form, THF - tetrahydrofuran form,

[b] Purities estimated from ^{1}H NMR data.

Mixed dFA and tFA standard compounds were separated and scanned over a wide m/z range in both positive and negative ionization mode. By using rather standard ionization parameters the negative ionization mode provided a more complete view of the standard compounds (**Figure 7**).

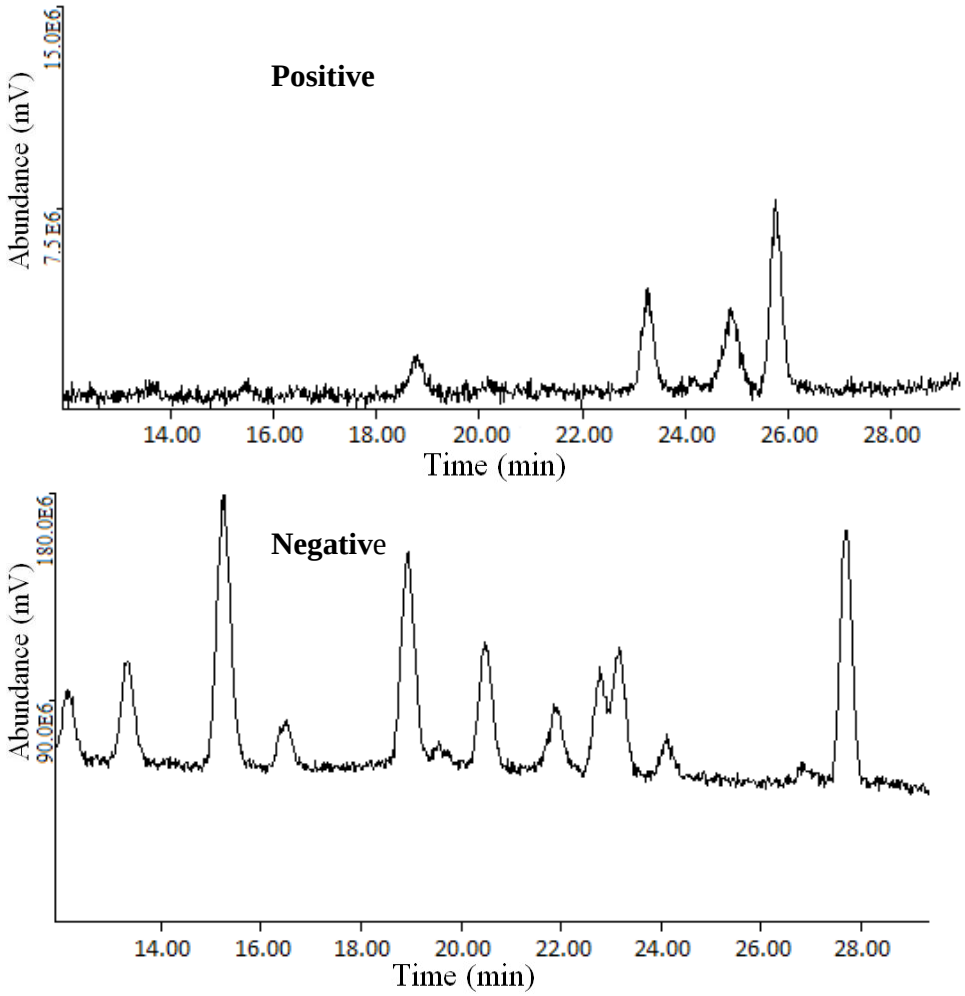

Figure 7. Mixed dehydrodiferulic acids (dFAs) and dehydrotriferulic acids (tFAs) monitored in positive and negative ionization mode. Chromatograms of a mixed diferulic acid and triferulic acid standard solution monitored by mass spectrometry in the positive (top) and negative (bottom) ionization mode

While positive ionization did not yield a promising chromatogram in this preliminary analysis this does not necessarily eliminate the use of positive ionization for this set of compounds. Positive ionization of these compounds may be best performed using a

solvent system buffered with salts which may promote the ionization of the dFAs and tFAs but was not further pursued as part of this study. In addition, the MS ionization parameters could be adjusted to optimize detection and this also was not undertaken in this prescreening using standard ionization parameters. Negative ionization parameters were optimized as such that predominantly quasi-molecular ions $[M-H]^-$ rather than fragment ions were generated.

The published elution order of the dFAs and tFAs [43]was confirmed and relative retention times were determined (**Table 3**).

Table 3. Relative retention times and correction factors of dehydrodiferulic acids (dFAs) and dehydrotriferulic acids (tFAs) in relation to 5-5Me-dehydrodiferulic acid (Me – methylated)

dFAs[a] and tFAs[a]	relative retention time	correction factor
8-8c-dFA	0.42	2.87
8-8nc-dFA	0.45	5.77
8-8THF-dFA	0.45	1.89
8-5nc-dFA	0.47	1.48
8-5nc/8-O-4-tFA	0.60	3.17
8-5nc/5-5-tFA	0.63	3.14
5-5-dFA	0.63	1.39
8-8c/8-O-4-tFA	0.74	3.51
8-O-4-dFA	0.79	1.25
8-5c-dFA	0.84	1.29
5-5/8-O-4-tFA	0.95	1.59
5-5Me-dFA	1.00	1.00
8-O-4/8-O-4-tFA	1.15	4.01
8-5dc-dFA	1.22	4.18

[a] c - cyclic form, nc - open (non-cyclic) form, THF - tetrahydrofuran form

These reflect a minor adjustment of the HPLC gradient of the previously developed HPLC/UV methodology which was necessary in order to reduce partial coelution of 8-8nc-, 8-8c-, 8-8THF- and 8-5nc-dFAs. A better separation was achieved by reducing the rate of methanol introduction into the gradient. However, coelution of 5-5-dFA and 8-5nc/5-5-tFA could not be addressed by this modification. The coelution of these compounds was not previously discovered in the UV-method since 8-5nc/5-5-tFA was not included in those analyses. Because a complete separation of these analytes could not be achieved, identification and quantification was based on the different molecular masses of these compounds. For quantification purposes the quasi molecular ion $[M-H]^-$ was used to develop SIR methods improving both sensitivity and selectivity as compared to full scan acquisition. As noted in the methods section the standard compounds were monitored using SIR on two channels with differing dwell times (**Figure 8**).

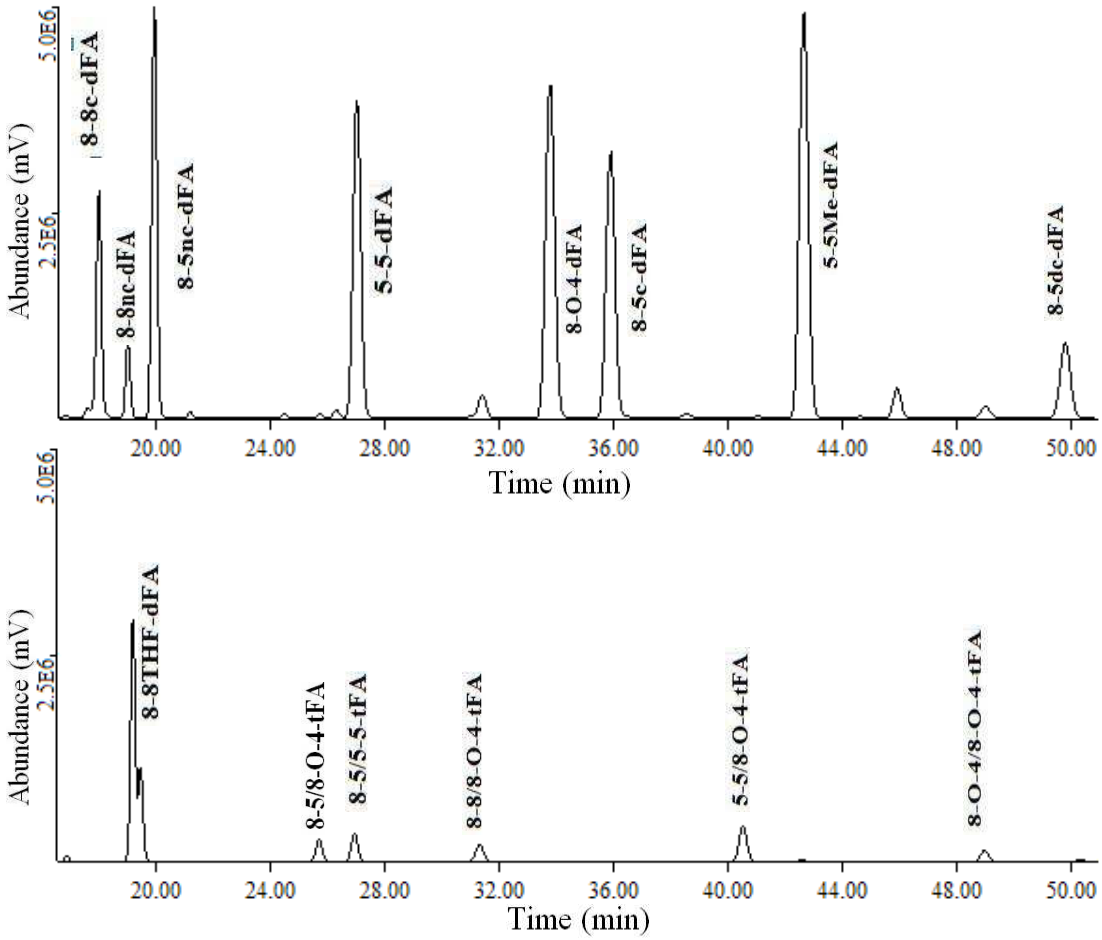

Figure 8. Select ion recording of dehydrodiferulic acids (dFAs) and dehydrotriferulic acids (tFAs) on two channels. Chromatograms showing the monitoring of a) 8-8c-, 8-8nc-, 8-5nc-, 5-5-, 8-O-4-, and 8-5c-dehydrodiferulic acids (m/z 385 $[M-H]^-$), 8-5dc-dehydrodiferulic acid (m/z 341 $[M-H]^-$) and 5-5Me-dehydrodiferulic acid (m/z 399 $[M-H]^-$) in channel #1 and b) 8-8THF-dehydrodiferulic acid (m/z 403 $[M-H]^-$) and 8-5nc/8-O-4-, 8-5nc/5-5-, 8-8c/8-O-4-, 5-5/8-O-4-, and 8-O-4/8-O-4-dehydrotriferulic acids (m/z 577 $[M-H]^-$) in channel #2 (c – cyclic, dc – decarboxylated, nc – noncyclic, Me – methylated, THF – tetrahydrofuran)

The grouping of masses allowed for the differentiation of coeluting 5-5-dFA and 8-5nc/5-5-tFA based on their $[M-H]^-$ (m/z 385 for 5-5-dFA, m/z 577 for 8-5nc/5-5-tFA). Grouping also allowed for the differentiation of 8-8THF-dFA (m/z 403 for $[M-H]^-$) from 8-8nc-dFA (m/z 385 $[M-H]^-$). Finally, increased specificity provided by the SIR method reduced the influence of coextracted matrix compounds on the chromatogram baseline, increasing accuracy and simplifying signal integration. As can be seen in (**Figure 8**), 8-8THF-dFA surprisingly showed a peak shape with two apexes which may indicate the presence of isomers of 8-8THF-dFA or that the injection solvent was too unpolar. However, since redissolution of dFAs and tFAs after drying is generally an issue the polarity of the injection solvent could not be increased.

Validation of the High Performance Liquid Chromatography-Mass Spectrometry Method. Examination of the validation parameters (**Table 4**) determined for the basic and matrix calibration reveals adequate method linearity as measured by correlation coefficients ranging from 0.9950 to 0.9999 as well as by visual inspection of the residual plots.

Table 4. Validation parameters for the basic and matrix calibration of dehydrodiferulic acids (dFAs)a and dehydrotriferulic acids (tFAs)a in the concentration ranges tested.

	range tested (µg/mL)	calibration equation (linear model)	correlation coefficients	LOD[b] (µg/mL)	LOQ[c] (µg/mL)	CV (BC)[d] (%)	CV (MC)[e] (%)	recovery[f] (%)
8-8c-dFA	3.0-30.0	y = 20222x + 7275.5	0.9988	0.04	0.08	8.7	3.8	70
8-8nc-dFA	3.0-30.0	y = 8577.3x + 6455.7	0.9999	0.26	0.85	5.8	4.0	76
8-8THF-dFA	3.0-30.0	y = 24621x + 13541	0.9997	0.03	0.05	6.4	4.2	84
8-5nc-dFA	3.0-30.0	y = 31111x + 38227	0.9950	0.02	0.051	9.3	4.3	86
8-O-4-dFA	3.0-30.0	y = 15748x + 221.04	0.9984	0.03	0.06	8.8	3.8	90
8-5c-dFA	3.0-30.0	y = 17651x + 1637.7	0.9969	0.03	0.07	7.7	2.6	92
5-5-dFA	3.0-30.0	y = 31964x + 41634	0.9978	0.34	1.03	8.0	2.8	92
5-5Me-dFA	3.0-30.0	y = 12702x + 3549.3	0.9996	0.02	0.051	7.5	3.2	92
8-5dc-dFA	3.0-30.0	y = 37292x - 10647	0.9963	0.05	0.20	8.8	13.1	53
8-8c/8-O-4-tFA	0.5-5.0	y = 32392x + 45832	0.9993	0.03	0.06	4.6	4.6	77
8-5nc/5-5-tFA	0.5-5.0	y = 28031x + 449.27	0.9991	0.02	0.048	4.4	4.4	69
8-5nc/8-O-4-tFA	0.5-5.0	y = 45971x + 19921	0.9996	0.02	0.12	5.7	4.9	81
5-5/8-O-4-tFA	0.5-5.0	y = 9794.4x + 2958.9	0.9989	0.02	0.06	4.7	4.0	87
8-O-4/8-O-4 tFA	0.5-5.0	y = 18011x + 7216.3	0.9991	0.07	0.21	5.1	4.2	90

[a] c - cyclic form, nc - open (non-cyclic) form, THF - tetrahydrofuran form, dc - decarboxylated form; [b] limit of detection (LOD), [c] limit of quantitation (LOQ), [d] coefficients of variation (CV) for the basic calibration (BC), [e] coefficients of variation for the matrix calibration (MC), [f] % recovery rate calculated from the matrix calibration

The limit of detection (LOD) and limit of quantitation (LOQ) were determined to range from 0.02 to 0.07 µg/mL and from 0.05 to 0.21 µg/mL, respectively, for most dFAs and tFAs. 8-8nc- and 5-5-dFA had, however, higher LOD and LOQ values of (0.26, 0.34 µg/mL) and (0.85, 1.03 µg/mL), respectively. Where comparable, these values are lower than the LOD/LOQ values published in the previously developed HPLC-UV methodology providing an increase in sensitivity by almost a factor of ten [43]. The coefficients of variation (CV) of the procedure for the basic calibration ranged between 4.4 and 9.3 % and are within the acceptable range of up to 10%, as was also observed for validation of the HPLC/UV method [43]. matrix calibration CV were between 2.6 and 4.9 % with the exception of 8-5dc which had a CV of 13.1% for the matrix calibration. This contrasts the HPLC-UV method which did not meet acceptable validation criteria for the matrix calibration with CVs ranging from 7.6 to 80%. Comparable or lower CVs observed in the HPLC-MS matrix calibration than the basic calibration suggests little or no interference of the matrix components with the analytes during this analysis largely due to the use of SIR methods. However, it has to be kept in mind that lignin was not added to the samples for the matrix calibration performed here since this method was developed for the analysis of low lignin samples such as cereal and pseudo-cereal flours. While the method has overall adequate CVs the recovery rates for five of the fourteen dFAs and tFAs analyzed by this methodology do not have demonstrated recovery rates in the desirable 80 to 110% range. The recovery rates for 8-8nc-dFA, 8-8c-dFA, 8-8c/8-O-4-tFA and 8-5nc/5-5-tFA were between 69% and 79% with 8-5dc-dFA having a low 53%

recovery. Overall, however, the recovery rates were better than those for the UV method. The recovery rates for the HPLC/UV method were in the acceptable range for some analytes, but 8-8THF-dFA with 76%, 8-5dc-dFA with 62%, 8-5c-dFA with 53%, 5-5/8-O-4-tFA with 45% and 8-8c/8-O-4-tFA with 34% had lower recoveries. The lower recovery rates noted for some dFAs and tFAs and the increased CV of the matrix calibration for 8-5dc-dFA may be in part due to incomplete extraction and redissolving of dried sample residues. Redissolving the dFAs and particularly tFAs has been observed to be difficult during the isolation and purification of the standard compounds and during previous analysis [43]. The overall slightly improved recovery rates found here may, however, indicate that dioxane/H_2O is a more suitable solvent for the redissolution of the extracted sample residues than THF/H_2O or methanol/H_2O which had been used previously [42, 43]. CFs were calculated against 5-5Me-dFA as described in the materials and methods section (**Table 3**). The CFs for 8-8nc-, 8-5dc-dFA and the trimers (except 5-5/8-O-4) are relatively high. Synthesis of a separate internal standard for the tFAs which is more closely related in molecular weight may also be worthwhile if tFAs are the primary focus of future studies.

Application of the Developed Method to Flour Samples. Contents of dFAs and tFAs were determined in six cereal (maize, wheat, rye, oat, barley and popcorn) whole grain flours where ferulates are ester-linked to arabinoxylans [1]. In addition, two pseudocereal whole grain flours, amaranth and buckwheat, were analyzed for their dFA and tFA contents. Whereas ferulates are ester-linked to pectic arabinans and galactans in amaranth [59], the

interactions of ferulates and cell wall polymers have not yet been studied in detail for buckwheat.

Table 5. Concentration of dehydrodiferulic acids (dFAs)a and dehydrotriferulic acids (tFAs)a in whole grain flours from different cereal and pseudocereals.

	Popcorn	Maize	Rye	Barley	Oat	Wheat	Buckwheat	Amaranth
Σ dFA (µg/g)	1528	473	470	332	287	258	50	53
8-8c (% dFA)	11	16	10	14	12	14	3	27
8-8nc (% dFA)	5	4	6	4	6	6	5	12
8-8THF (% dFA)	14	6	4	23	23	4	1	2
Σ8-5 (% dFA)	33	35	56	32	34	45	91	31
5-5 (% dFA)	17	16	10	10	9	11	+[b]	10
8-O-4 (% dFA)	16	16	11	12	13	14	1	9
Σ tFA (µg/g)	161	43	27	24	23	25	1	3
8-5nc/8-O-4 (% tFA)	12	14	15	15	14	15	9	14
8-5nc/5-5 (% tFA)	14	18	17	14	16	15	-[c]	17
8-8c/8-O-4 (% tFA)	18	16	12	15	14	12	-	16
5-5/8-O-4 (% tFA)	38	34	32	27	26	29	19	24
8-O-4/8-O-4 (% tFA)	19	19	24	29	30	29	73	30

[a] c - cyclic form, nc - open (non-cyclic) form, THF - tetrahydrofuran form;

[b] detected but not quantifiable, [c] not detected

As discussed in more detail elsewhere [1], 8-5c-, 8-5nc- and 8-5dc-dFAs presumably have the same precursor in the plant, esterified 8-5c-dFA. Therefore these dimers, although analyzed individually, should not be discussed independently but as the sum of 8-5-coupled dimers (8-5-dFA).

In maize and popcorn, a special maize variety, 8-5-dFA was the dominant dFA followed by 5-5-dFA, 8-O-4-dFA and the 8-8-coupled dimers, 8-8c-, 8-8THF- and 8-8nc-dFA

(**Table 5, Figure 9 A,B**). This pattern is roughly comparable to previous analyses of maize bran and whole grain using HPLC/UV and GC-MS methodologies although previous studies did not necessarily determine all dimers which were analyzed here [40, 43, 58, 60]. Popcorn contained a higher proportion of 8-8THF than maize which contained 8-8c-dFA as the dominant 8-8-coupled dimer. Different from maize, other cereals generally have lower proportions of 5-5-dFA. This can be confirmed in this study for rye and wheat which also contained 8-5-dFA as the dominant dFA but followed by 8-O-4-dFA. In wheat, the proportion of 5-5-dFA was even lower than the proportion of 8-8c-dFA. These data match previous analyses by Dobberstein and Bunzel, Bunzel and co-workers and Garcia-Conesa and co-workers [40, 53, 61] while Andreasen and co-workers as well as Gallardo and co-workers reported a lower 8-5-dFA ratio [58, 62-64]. Barley and oats showed a distinct dFA composition with much larger 8-8THF-dFA portions (23%) than the other flours demonstrating the underestimation of ferulate dimers by not including this more recently found dimer [65]. However, 8-5-dFA dominated also in these samples. Ferulate dimers were identified in both pseudocereal flours, too. An unusually large portion (27% of the total dFAs) of 8-8c-dFA was found in amaranth whereas buckwheat contained more than 90% 8-5-dFA with small portions of 8-8nc-, 8-8c-, 8-8THF-, and 8-O-4-dFA only and an identifiable but not quantifiable amount of 5-5-dFA. Buckwheat was analyzed for monomeric HCAs and dFAs in a previous study but dFAs were not detected [64]. The association between ferulates and cell wall polysaccharides in amaranth has been confirmed but studies quantifying diferulates in literature are lacking [59].

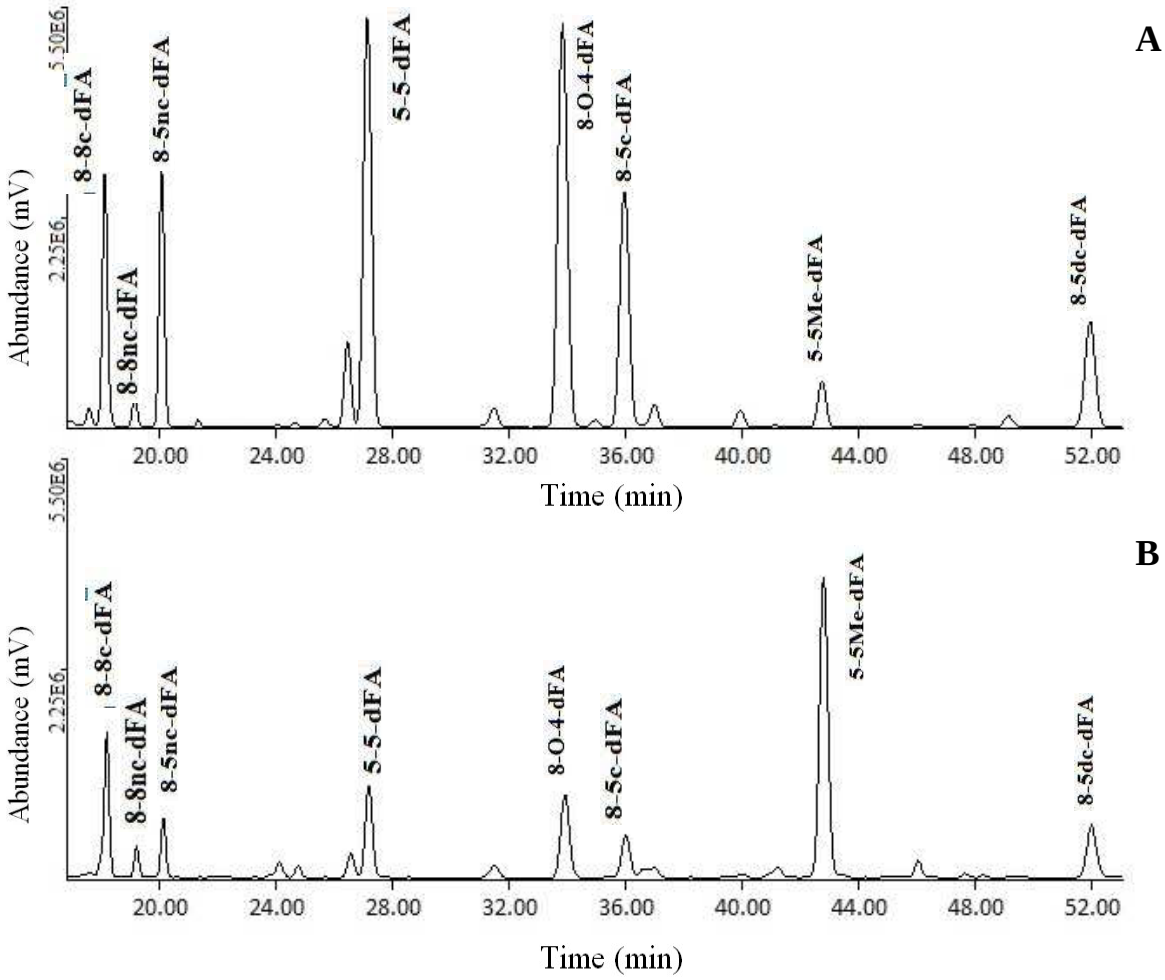

Figure 9. Analysis of dehydrodiferulic acids (dFAs) and dehydrotriferulic acids (tFAs) in popcorn and amaranth. Chromatograms (channel #1) obtained from the analysis of dehydrodiferulic acids (with the exception of 8-8THF-dehydrodiferulic acid which is monitored in channel #2) in the alkaline hydrolyzates of A) popcorn diluted 1 to 10 in 50:50 dioxane:H_2O and B) amaranth whole grain flours; (THF – tetrahydrofuran)

Highest dFA contents were found in popcorn (ca. 1.5 mg/g flour) followed by maize, rye, barley, oats and wheat (ranging between ca. 250 to 475 μg/g flour), with amaranth and buckwheat containing much lower dFA levels (ca. 50 μg/g flour). These concentrations are roughly comparable to those found previously in flours or fiber samples obtained from whole grain flours [40, 43, 63]. To compare literature data from fiber samples, dietary fiber contents of 6 – 12%, depending on the cereal, need to be considered to calculate approximate dFA concentrations in the flours. Other studies which quantified dFAs in milling fractions or isolated cell walls rather than the whole grain also observed this pattern [51, 58, 60, 64, 66-68]. The large amounts of dFAs in popcorn were expected from previous studies using popcorn as a maize variety [40, 61]. Also, popcorn contains a higher ratio of pericarp to (hard) endosperm and as such whole grain flours of popcorn may contain higher levels of pericarp than maize commonly used for whole grain flours which have much larger amounts of (soft) endosperm [69]. Due to the primary location of these compounds in the pericarp, higher levels of dFAs and tFAs were expected in popcorn flour compared to maize flour. Also popcorn has been shown to have a highly crystallized pericarp and this may be due to increases in cross-linking by ferulates [70]. Data on the dFA contents of dictotyledenous plants are rare. While quantitative data regarding dFA contents in buckwheat and amaranth is lacking in literature, analyses of other dicots suggest that they contain lower levels of dFAs compared to cereals, which was also observed in this study [43, 71, 72].

5-5/8-O-4-tFA was the dominant tFA in maize, popcorn, and rye with lower proportions found for 8-O-4/8-O-4-, 8-8c/8-O-4-, 8-5nc/5-5-, and 8-5nc/8-O-4-tFA. For trimers

containing 8-5-linkages the same considerations as for the 8-5-linked dimers need to taken into account. Most likely only the 8-5c-linkage exists in the plant which is partially transformed into the 8-5nc-linkage followed by decarboxylation during alkaline hydrolysis. Therefore, the contents of these trimers might be higher in the flour; however, we do not have standard compounds to identify or quantify the corresponding 8-5-containing trimers. Wheat flour contained about equal proportions of 5-5/8-O-4- and 8-O-4/8-O-4-tFAs as predominant trimers and barley, oats, and amaranth were dominated by 8-O-4/8-O-4-tFA but still contained larger proportions of 5-5/8-O-4-tFA. Buckwheat overwhelmingly contained 8-O-4/8-O-4-tFA which was unexpected since 8-5-dFA dominated the dimer fraction. Unfortunately, studies regarding tFAs have currently been focused only on the use of 5-5/8-O-4 as a biomarker in milling fractions [57] and there are very few additional studies of the tFA patterns in cereals with which to compare these results [1, 43].

The tFA contents of the cereal flours are roughly about one tenth of the dFA contents ranging between 23 and 161 µg/g flour. Even lower amounts were found for the pseudocereal flours with 1 – 3 µg/g flour. Accordingly, popcorn contained the highest overall trimer content followed by maize, rye, wheat, barley and oats (**Table 5**). Two recent studies quantified some of the tFAs in maize and milling fractions of wheat grain [43, 57]. Dobberstein found the total tFAs (sum of 5-5/8-O-4-, 8-O-4/8-O-4- and 8-8c/8-O-4-tFA only) to be present in maize (popcorn) at 916 µg/g insoluble fiber. Barron et al. found the 5-5/8-O-4-tFA content to be 910 to 1670 µg/g in the outer pericarp of wheat and 20 to 60 µg/g in the whole grain. This value is larger than what we observed here. 5-

5/8-O-4-tFA accounted for 29% of the total tFA content resulting in approximately 7 µg 5-5/8-O-4-tFA per g whole grain wheat flour.

3.4 Conclusion

In conclusion, an HPLC-MS methodology was developed to separate and quantify dFAs and tFAs in low lignin plant materials such as cereal grains. This methodology demonstrated adequate selectivity, sensitivity, and precision (with the exception of the 8-5dc-dFA). However, accuracy while acceptable for most of the compounds needs improvement for some compounds, especially for accurate quantification of 8-5dc-dFA. Analysis of dFA and tFA contents demonstrated that dFA contents are largely underestimated by using methods which are not able to quantify the 8-8-dimers. Several methods are not able to quantify 8-8-dimers due to the coelution with late eluting monomers or by just ignoring the existence of a third 8-8-coupled dimer, the 8-8THF-dFA. This is the first study quantifying five previously identified ferulate trimers in different cereals and pseudocereals. The tFA contents are roughly one tenth of the contents of dFAs in these samples.

Chapter 4 Supplementary Data

4.1 Development of an High Performance Liquid Chromatography-Mass Spectrometry Method for the Analysis of Hydroxycinnamic Acids and other Plant Phenolic Monomers

An HPLC-MS method was also developed for the analysis of monomeric HCAs and other phenolic monomers in plant materials. This method utilized a different elution gradient to separate the phenolic monomers from dFAs and tFAs which might otherwise co-elute with the phenolic monomers. This approach was previously developed and validated by Dobberstein and Bunzel for an HPLC-UV method [43]. Instead of using UV detection, an MS detector coupled to the HPLC was used here. Validation parameters were assessed and the method was applied to plant materials.

Method development and validation and its application to plant materials were generally performed as described in Chapter 3 for the dFAs and tFAs. However, as already mentioned, the HPLC gradient used here was different from the gradient used for the separation of the dFAs and tFAs and the SIR MS method differed as well. HCAs and other plant phenolic monomers were separated at a flow rate of 1.2 mL/min using the following gradient: Initially 89% A/11% C, increased linearly over 10 min to 88% A/1% B/11% C, linear over 10 min to 82% A/3% B/15% C, linear over 20 min to 5% A/45% B/50% C and re-equilibrated at initial conditions (89% A/11% C) for 10 min. Analyte peaks were monitored through SIR separated into three channels. Monitored ions for the early eluting HCAs were 137 $[M-H]^-$ (4-HBA), 151 $[M-H]^-$ (4-HPAA), 179 $[M-H]^-$ (CA),

167 $[M-H]^-$ (VnA), 197 $[M-H]^-$ (SyA), 121 $[M-H]^-$ (4-HBAl) and 165 $[M-H]^-$ (4-HPPA) on channel #1, 151$[M-H]^-$ (Vn) on channel #2 and 119 $[M-COOH]^-$ (*trans* and *cis*-*p*CA), 181 $[M-H]^-$ (SyAl), 193 $[M-H]^-$ (*trans* and *cis*-FA), 223 $[M-H]^-$ (SA) and 163 $[M-H]^-$ (*o*-HCA) on channel #3 with a scan dwell time of 0.3 sec for channel #1 and #3 and 0.5 sec for channel #2. Finally, a different internal standard, which is structurally closer to the analytes than the 5-5Me-dFA standard used for dFA and tFA analysis, *o*-HCA, was used for the phenolic monomers.

The following phenolic monomers were included in the method (in order of elution on the phenyl hexyl column): 4-hydroxybenzoic acid (4-HBA), 4-hydroxyphenylacetic acid (4-HPAA), CA, vanillic acid (VnA), syringic acid (SyA), 4-hydroxybenzaldehyde (4-HBAl), 4-hydroxyphenylpropionic acid (4-HPPA), *trans*-*p*-coumaric acid (*trans*-*p*CA), vanillin (Vn), *cis*-*p*CA, syringaldehyde (SyAl), *trans*-ferulic acid (*trans*-FA), SA, *cis*-FA and *o*-HCA. Stock solutions of the phenolic monomers were prepared from commercially available standard compounds (Sigma Aldrich (St. Louis, MO), MP Biomedicals (Salon, OH) and Alfa Aesar (Ward Hill, MA)) with the exception of *cis*-pCA and *cis*-FA which were prepared form their *trans*-isomers by UV irradiation as described previously [43]. The stock solution mixture was analyzed by HPLC-MS using ESI operating in the negative mode with a capillary voltage of 4 kV, cone voltage of 30 V, source temperature of 150°C, desolvation temperature of 450°C, desolvation gas flow of 600 L/h and a gas flow through the cone at 75 L/h. Analysis of the standard mixture covered the m/z range of 100 to 800 amu and was performed in order to identify quasi molecular ions $[M-H]^-$ and fragment ions in the mass spectra of the phenolic monomers as described above for

the dFAs and tFAs. The published elution order of the phenolic monomers [43] was confirmed and relative retention times were determined (**Table 6, Figure 10**).

Table 6. Relative retention times and correction factors of phenolic monomers[a] to o-hydroxycinnamic acid (o-HCA) as internal standard

phenolic monomer	relative retention time	correction factor
4-HBA	0.23	3.26
4-HPAA	0.25	21.10
CA	0.29	3.90
VnA	0.31	16.61
SyA	0.35	19.18
4-HBAl	0.38	0.86
4-HPPA	0.44	9.85
trans-pCA	0.53	2.33
Vn	0.54	7.55
cis-pCA	0.59	1.64
SyAl	0.66	50.91
trans-FA	0.72	10.77
SA	0.77	17.33
cis-FA	0.80	6.26
o-HCA	1.00	1.00

[a] 4-hydroxybenzoic acid (4-HBA), 4-hydroxyphenylacetic acid (4-HPAA), caffeic acid (CA), vanillic acid (VnA), syringic acid (SyA), 4-hydroxybenzaldehyde (4-HBAl), 4-hydroxyphenylpropionic acid (4-HPPA), *trans*-*p*-coumaric acid (*trans*-*p*CA), vanillin (Vn), *cis*-*p*-coumaric acid (cis-*p*CA), syringaldehyde (SyAl), *trans*-ferulic acid (*trans*-FA), sinapic acid (SA), *cis*-ferulic acid (*cis*-FA), o-hydroxycinnamic acid (*o*-HCA).

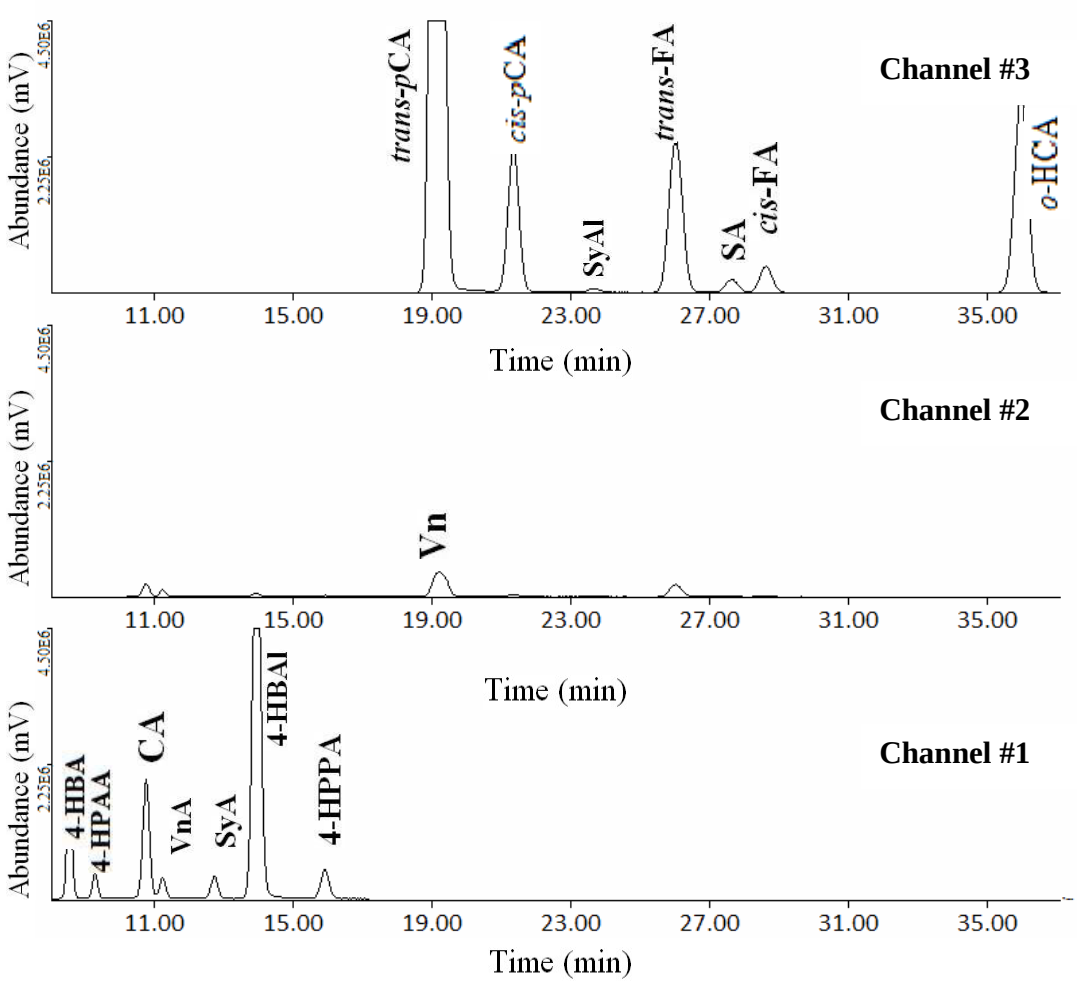

Figure 10. Select ion recording of phenolic monomers separated by reverse phase-high performance liquid chromatography (RP-HPLC) on three channels. Abbreviations: 4-hydroxybenzoic acid (4-HBA), 4-hydroxyphenylacetic acid (4-HPAA), caffeic acid (CA), vanillic acid (VnA), syringic acid (SyA), 4-hydroxybenzaldehyde (4-HBAl), 4-hydroxyphenylpropionic acid (4-HPPA), trans-p-coumaric acid (trans-pCA), vanillin (Vn), cis-pCA, syringaldehyde (SyAl), trans-ferulic acid (trans-FA), sinapic acid (SA), cis-FA and o-hydroxycinnamic acid (o-HCA).

4.2 Validation of a High Performance Liquid Chromatography-Mass Spectrometry Method for the Analysis of Hydroxycinnamic Acids and other Plant Phenolic Monomers

Basic and matrix calibration were performed in order to determine the accuracy and precision of the method. Both calibrations were performed in triplicate as described for the dFAs and tFAs. The tested concentration range for most phenolic monomers was 2.5 to 12.5 µg/mL (steps 2.5, 5.0, 7.5, 10.0 and 12.5 µg/mL). Because they are much more prominent in most plant materials than the other phenolic monomers, *trans*-*p*CA and *trans*-FA were calibrated in a higher concentration range of 25.0 to 125.0 µg/mL (steps 25.0, 50.0, 75.0, 100.0 and 125.0 µg/mL). Examination of the validation parameters (**Table 7**) determined for the basic and matrix calibration revealed adequate method linearity as measured by correlation coefficients ranging from 0.9935 to 0.9998 as well as by visual inspection of the residual plots. The LOD and LOQ values were determined to range from 0.04 to 0.39 µg/mL and from 0.08 to 0.78 µg/mL, respectively, for most phenolic monomers. SyAl had, however, higher LOD and LOQ values of 1.25 and 5.0 µg/mL, respectively. Where comparable, these values are slightly higher than the LOD/LOQ values published in the previously developed HPLC-UV methodology [43]. The CV of the procedure for the basic calibration ranged from 0.8 to 37% and 0.9 to 51% in the matrix calibration, with 4-HPAA, SyA, SyAl, VnA, *trans*-FA and SA falling outside of the acceptable CV of 10% or less. VnA, *trans*-FA and SA demonstrated adequate recovery suggesting that liquid-liquid sample extraction and re-dissolution of

the sample extract may not be the most likely source of variation. The choice of the quasi molecular ion for these compounds may not be the most stable ion for detection. Recovery rates of phenolic monomers ranged from 63 to 93%, with 4-HPAA, SyA, SyAl and CA being below the acceptable 80% recovery level. CA meets adequate levels of variation but failed to meet an adequate recovery level suggesting that incomplete extraction or re-dissolution may be the cause of decreased recovery observed with this compound. 4-HPAA in particular was also found to have a lowered rate of recovery in the previously validated HPLC-UV methodology [43] and it was suggested that 4-HPAA may be more susceptible to interference from lignin degradation products. This should not be an issue for this investigation as lignin was not included in the matrix calibration. 4-HPAA, SyA and SyAl failed to meet acceptable levels of either CV or recovery. The source of variation and reduced recovery observed for the aforementioned phenolic monomers has not yet been determined. However, incomplete sample extraction, incomplete dissolution of the sample extract and selection of the quasi molecular ion as the detection monitoring ion; may be responsible for the variation observed here.

CFs were calculated against *o*-HCA as described for the dFAs and tFAs in the materials and methods section (**Table 6**). The CFs for most of the phenolic monomers are relatively high with the exception of *trans-p*CA, *cis-p*CA, CA, 4-HBA and 4-HBAl. This suggested that *o*-HCA may not be the most suitable internal standard and may also suggest that quantification using the quasi molecular ion may not be the best approach.

Table 7. Validation parameters for the calibration of phenolic monomersa in the concentration ranges tested

	range tested (µg/mL)	calibration equation (linear model)	correlation coefficients	LOD[b] (µg/mL)	LOQ[c] (µg/mL)	CV (BC)[d] (%)	CV (MC)[e] (%)	recovery[f] (%)
4-HBA	2.5-12.5	y = 42859x + 37227	0.9998	0.08	0.16	3.4	3.3	91.3
4-HPAA	2.5-12.5	y = 8910.4x - 1350.2	0.9985	0.31	1.25	17.3	21.1	75.3
CA	2.5-12.5	y = 48507x + 29498	0.9998	0.16	0.31	3.1	3.9	70.7
VnA	2.5-12.5	y = 9454x - 349.54	0.9981	0.31	1.25	16.2	16.6	83.9
SyA	2.5-12.5	y = 10474x + 619.08	0.9985	0.31	0.63	14.5	19.2	68.3
4-HBAl	2.5-12.5	y = 181222x + 178878	0.9996	0.04	0.08	0.8	0.9	86.3
4-HPPA	2.5-12.5	y = 16051x - 5298.4	0.9982	0.31	1.25	9.8	9.9	92.3
trans-pCA	25.0-125.0	y = 55908x + 1E+06	0.9946	<0.39	<0.39	2.4	2.3	90.9
Vn	2.5-12.5	y = 19437x + 10656	0.9988	0.16	1.25	7.7	7.6	85.7
cis-pCA	2.5-12.5	y = 95045x + 24336	0.9994	0.08	0.16	1.6	1.6	87.4
SyAl	2.5-12.5	y = 4251.3x - 2507.9	0.9935	1.25	5.00	36.6	50.9	62.6
trans-FA	25.0-125.0	y = 12428x + 285595	0.9960	0.39	0.78	10.8	10.8	96.8
SA	2.5-12.5	y = 10197x + 10333	0.9987	0.31	1.25	14.0	17.3	80.8
cis-FA	2.5-12.5	y = 24952x + 9100	0.9997	0.16	0.31	6.0	6.3	87.2
o-HCA	2.5-12.5	y = 149874x + 59905	0.9993	0.08	0.16	1.0	1.0	92.5

[a]4-hydroxybenzoic acid (4-HBA), 4-hydroxyphenylacetic acid (4-HPAA), caffeic acid (CA), vanillic acid (VnA), syringic acid (SyA), 4-hydroxybenzaldehyde (4-HBAl), 4-hydroxyphenylpropionic acid (4-HPPA), *trans*-*p*-coumaric acid (*trans*-*p*CA), vanillin (Vn), *cis*-*p*-coumaric acid (*cis*-*p*CA), syringaldehyde (SyAl), *trans*-ferulic acid (*trans*-FA), sinapic acid (SA), *cis*-ferulic acid (*cis*-FA) and *o*-hydroxycinnamic acid (*o*-HCA); [b] limit of detection (LOD), [c] limit of quantitation (LOQ), [d] coefficients of variation (CV) for the basic calibration (BC), [e] coefficients of variation for the matrix calibration (MC), [f] % recovery rate calculated from the matrix calibration

4.3 Application of Phenolic Monomers Method to Flour Samples

Sample extracts were separated and quantified using an HPLC coupled to single quadrupole mass spectrometer as described in chapter 4.1. If necessary, sample extracts were diluted in H_2O/dioxane 50/50 (v/v) to stay within the tested concentration range for the analytes.

Table 8. Concentrations of phenolic monomersa in whole grain flours from different cereals and pseudocereals

	Popcorn µg/g	Maize µg/g	Rye µg/g	Wheat µg/g	Barley µg/g	Oat µg/g	Amaranth µg/g	Buckwheat µg/g
4-HBA	90	42	42	35	48	57	64	130
4-HPAA	34	21	15	15	24	15	10	11
CA	79	24	34	19	40	93	6	28
VnA	29	10	12	13	10	18	31	6
SyA	31	23	8	15	15	21	-	-
4-HBAl	8	3	3	1	3	4	6	5
4-HPPA	-	-	-	-	-	-	-	-
trans-pCA	637	134	91	29	143	50	21	72
Vn	51	8	6	3	7	7	1	7
cis-pCA	3	2	10	2	28	1	3	4
SyAl	42	12	10	6	-	13	-	-
trans-FA	2685	633	513	419	417	366	130	26
SA	401	247	244	141	38	116	4	49
cis-FA	80	20	80	58	87	11	15	1

[a]4-hydroxybenzoic acid (4-HBA), 4-hydroxyphenylacetic acid (4-HPAA), caffeic acid (CA), vanillic acid (VnA), syringic acid (SyA), 4-hydroxybenzaldehyde (4-HBAl), 4-hydroxyphenylpropionic acid (4-HPPA), *trans*-*p*-coumaric acid (*trans*-*p*CA), vanillin (Vn), *trans*-*p*-coumaric acid (*cis*-*p*CA), syringaldehyde (SyAl), *trans*-ferulic acid (*trans*-FA), sinapic acid (SA), *cis*-ferulic acid (*cis*-FA) and *o*-hydroxycinnamic acid (*o*-HCA); (-) not detected

Phenolic monomer contents were determined in six cereals and two pseudocereals as described for the dFAs and tFAs (**Table 8**). Overall, phenolic monomer contents were greater in the cereals than the pseudocereals (**Figure 11**) with ferulic acid contents similar to those found in literature [27]. As expected, *trans*-FA was the dominant phenolic monomer followed by *trans*-pCA among the cereals and amaranth. 4-HBA was found to be the dominant phenolic monomer in buckwheat which had the lowest overall phenolic monomer content. However, since the validation data for most of the phenolic monomers did not meet our acceptance criteria these data can be considered as semiquantitative data at best.

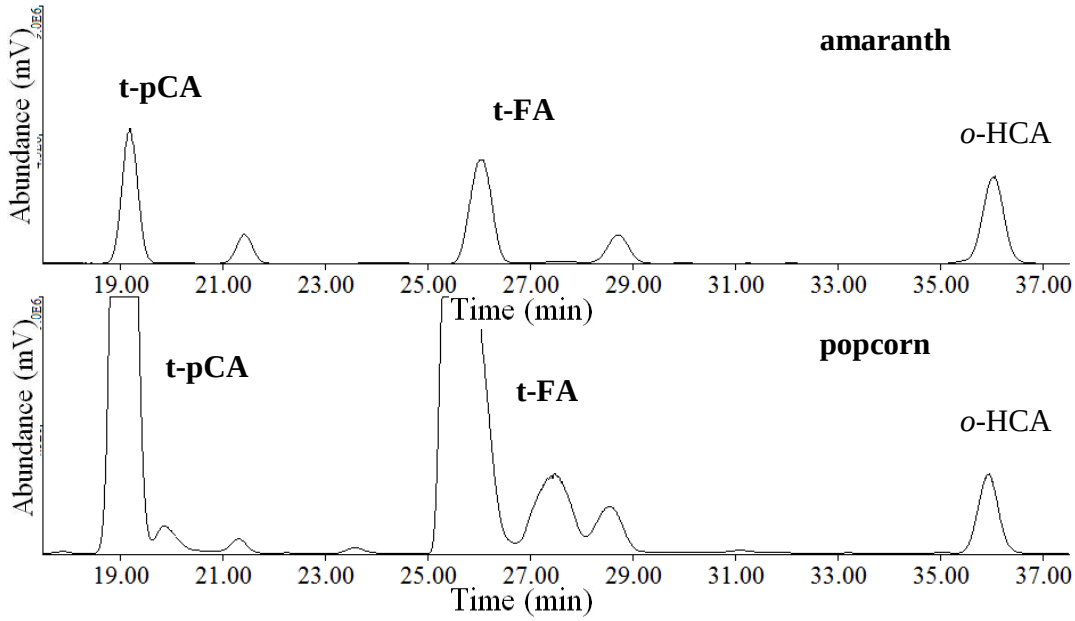

Figure 11. Analysis of popcorn and amaranth for phenolic monomers. Later eluting phenolic monomers in amaranth (top) and in popcorn (bottom).

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Chapter 6 Appendix

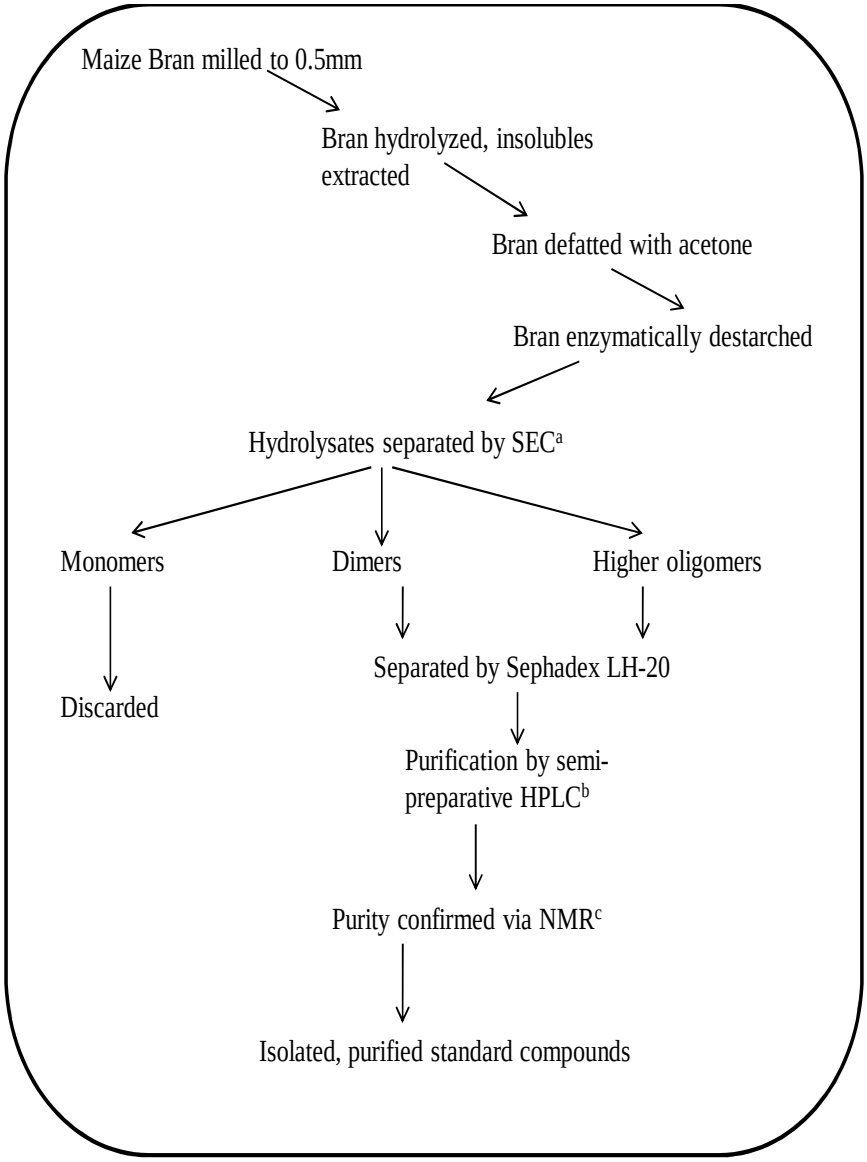

Figure 12. Isolation of standard compounds (dehydrodiferulic and dehydrotriferulic acids) from maize bran. a Size exclusion chromatography (SEC), b high performance liquid chromatography (HPLC), c nuclear magnetic resonance (NMR).

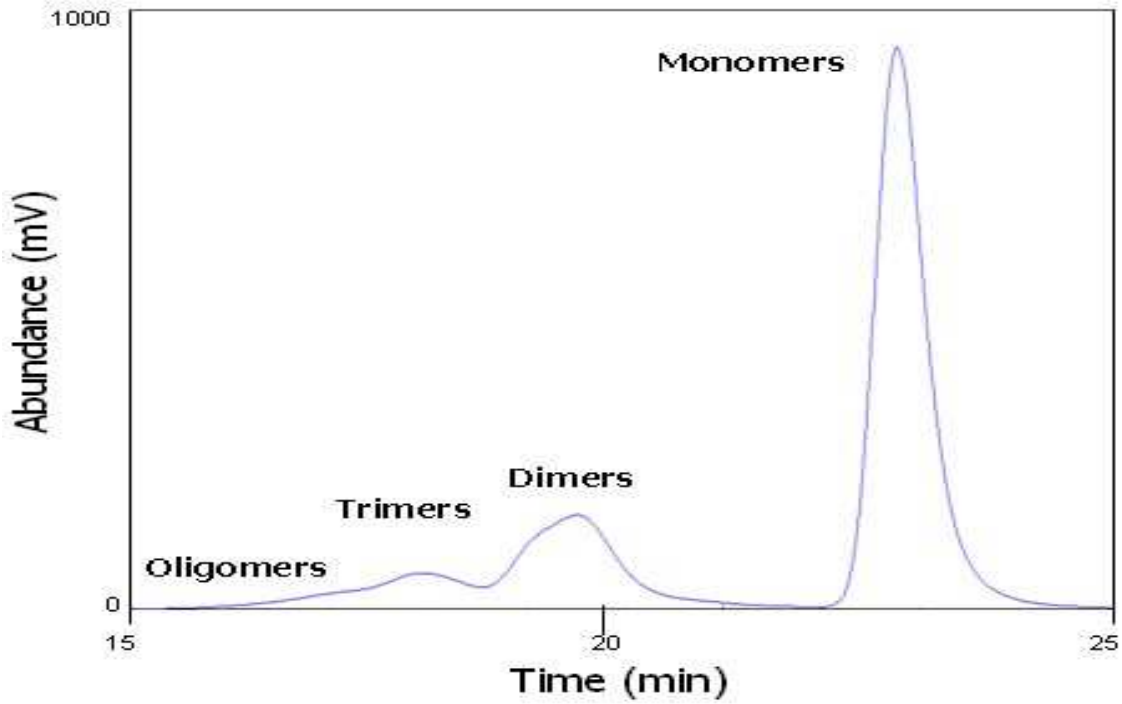

Figure 13. Size exclusion chromatogram of alkaline hydrolysates from destarched maize bran (UV detection at 280 nm)

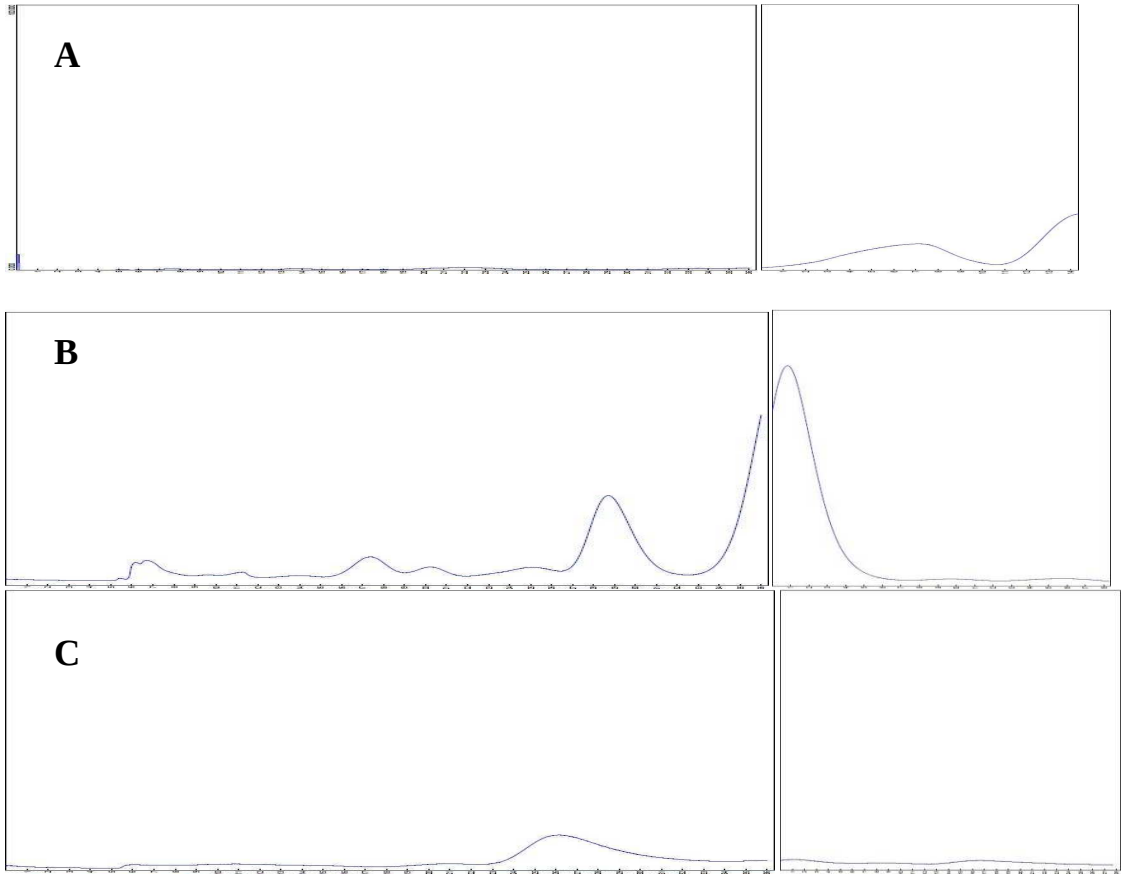

Figure 14. Separation of phenolic dimers obtained from size exclusion chromatography of maize bran hydrolysates on Sephadex LH-20 using a three step elution; A) elution #1, 0.5 mM TFA/MeOH (95/5, v/v), 1.5 mL/min, 42 hours; B) elution #2, 0.5 mM TFA/MeOH (50/50, v/v), 1.0 mL/min, 48 hours and C) elution #3 0.5 mM TFA/MeOH (40/60, v/v), 1.0 mL/min, 55 hours.

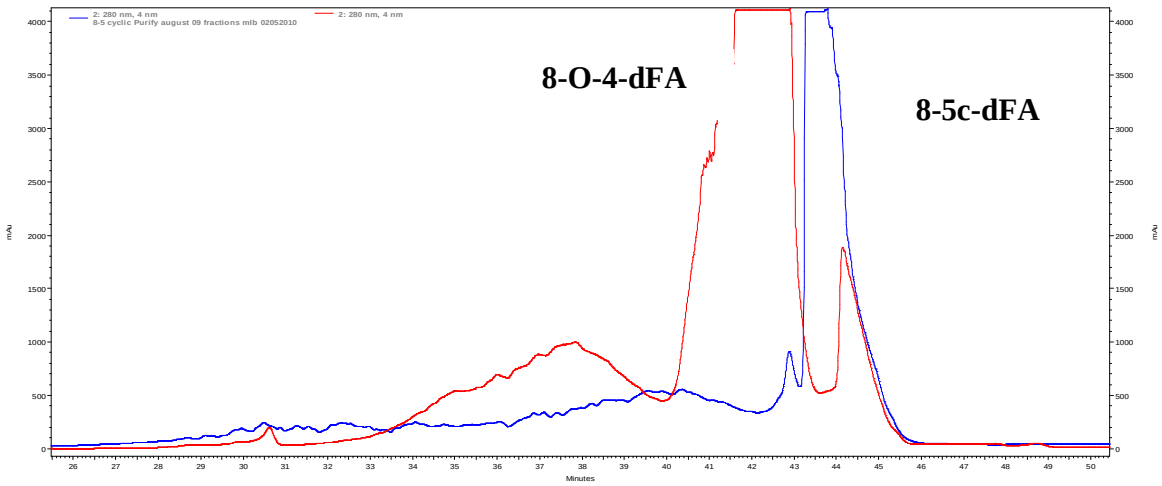

Figure 15. Semi-preparative high performance liquid chromatography (HPLC) purification of dehydrodiferulic acids (dFAs). a) 8-O-4-dFA and b) 8-5cyclic(c)-dFA.

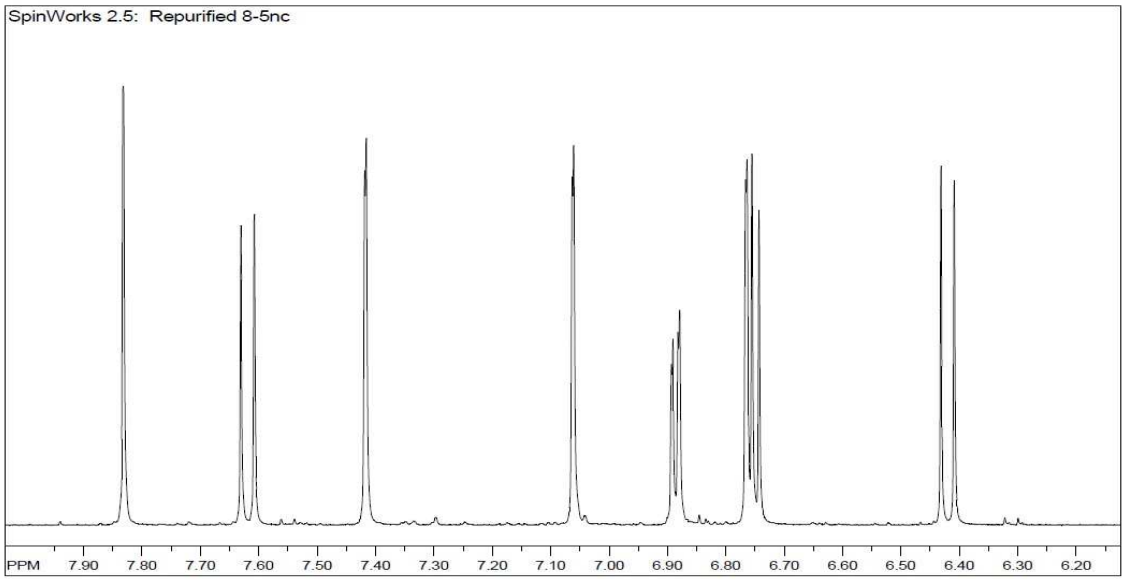

Figure 16. Example for the determination of standard compound purity by nuclear magnetic resonance spectra as demonstrated for 8-5non-cyclic-dehydrodiferulic acid.

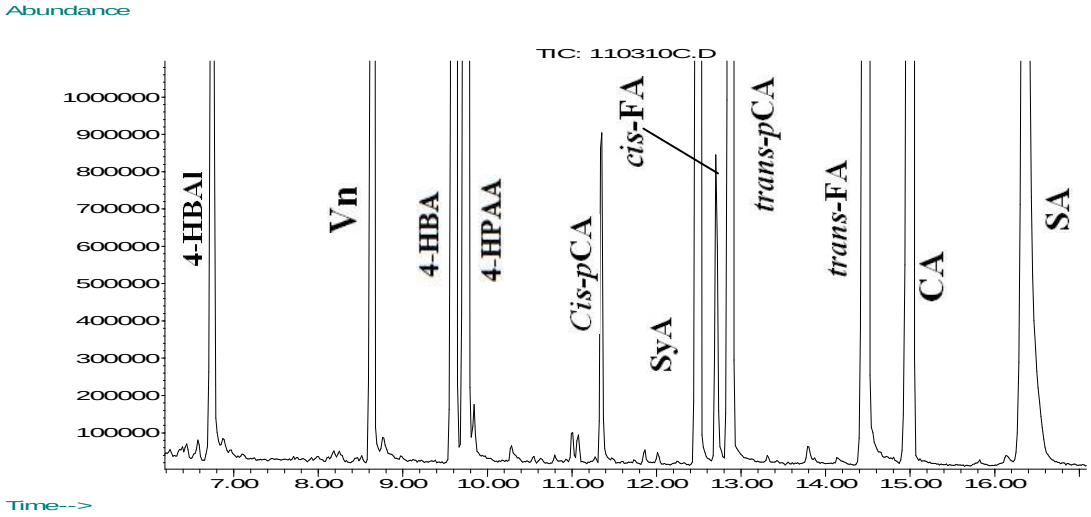

Figure 17. Separation of phenolic monomers using high temperature gas chromatography mass spectrometry (GC-MS). Abbreviations: 4-hydroxybenzoic acid (4-HBA), 4-hydroxyphenylacetic acid (4-HPAA), caffeic acid (CA), syringic acid (SyA), 4-hydroxybenzaldehyde (4-HBAl), trans-p-coumaric acid (trans-pCA), vanillin (Vn), cis-p-coumaric acid (cis-pCA), trans-ferulic acid (trans-FA), sinapic acid (SA) and cis-ferulic acid (cis-FA).

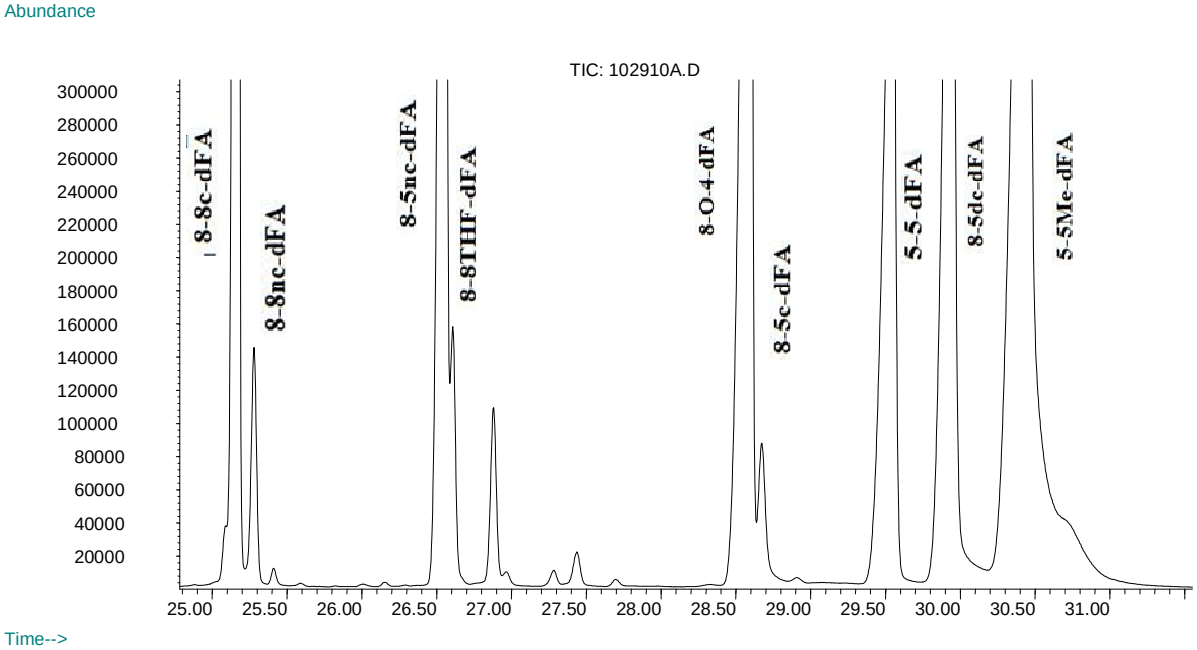

Figure 18. Separation of dehydrodiferulic acids (dFAs) using high temperature gas chromatography-mass spectrometry (GC-MS). Abbreviations: a c - cyclic form, nc - open (non-cyclic) form, THF - tetrahydrofuran form, dc - decarboxylated form

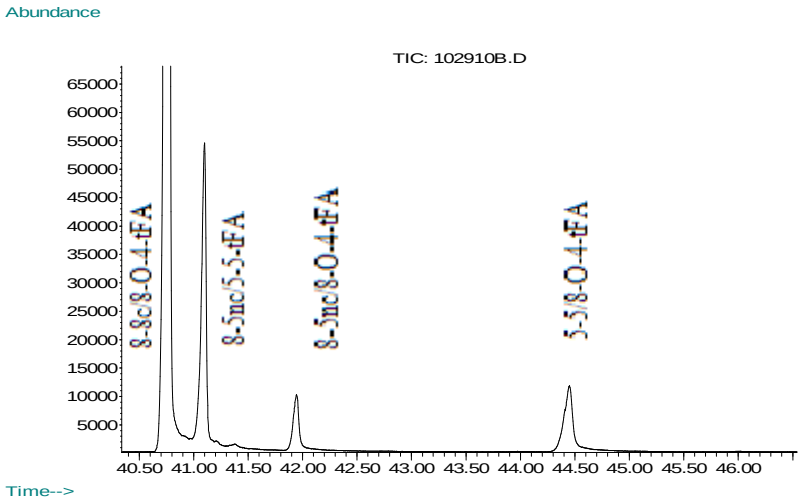

Figure 19. Separation of dehydrotriferulic acids (tFAs) using high temperature gas chromatography-mass spectrometry (GC-MS). Abbreviations: a c - cyclic form, nc - open (non-cyclic) form

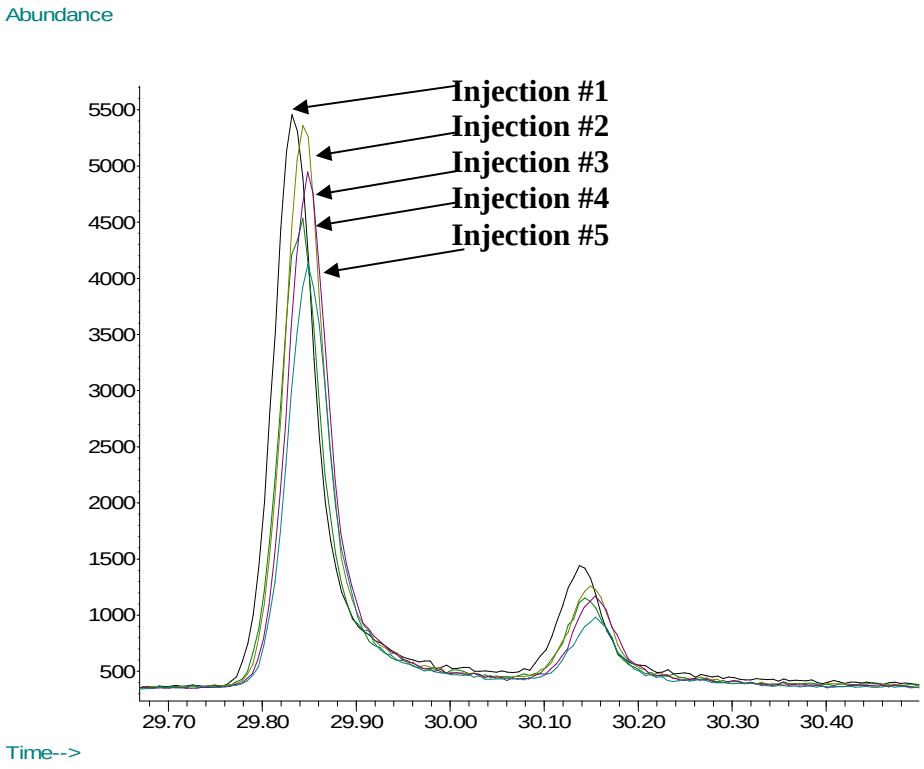

Figure 20. Signal drift observed during the sequential analysis of dehydrodiferulic acids (dFAs) by gas chromatography-mass spectrometry (GC-MS)

Table 9. Gas chromatography-mass spectrometry (GC-MS) method conditions for dehydrodiferulic acids (dFAs) and dehydrotriferulic acids (tFAs) analysis.

GC Conditions	
Column	Restek 30m MXT-5(5% phenyl 95% dimethylpolysiloxane)
Internal Diameter (I.D.)	0.25 mm
Film Thickness	0.25 µm
Inlet Temperature	400°C
Transfer Line Temperature	400°C
Injection Mode	Pulsed splitless (26 psi for 0.75 min)
Injection Volume	2 µL
Carrier Gas Flow	Heliun at constant flow (1.0 mL/min)
Oven Program	
Initial Temperature	100°C (hold 1 min)
Ramp #1	10°C/min to 248°C
Ramp #2	30°C/min to 300°C (hold 15 min)
Ramp #3	40/min to 350°C (hold 15 min)
Ramp #4	40°C/min to 400°C (hold 10 min)
MS Conditions	
Acquisition Parameters	Electron impact (EI), select ion monitoring (SIM)
Sampling Rate	1 (scan rate at 1.53 scans/sec)
Threshold	150
Filament Delay	4.0 min
MS Temp	
Ion Source	230°C
Quadrupole	150°C

Table 10. Validation parameters for the basic calibration dehydrodiferulic acids (dFAs) a and dehydrotriferulic acids (tFAs) a using gas chromatography-mass spectrometry (GC-MS) in the concentration ranges tested.

	range tested (µg injected)	calibration equation (linear model)	correlation coefficients	CV (BC)[b] %
8-8c-dFA	0.1-0.35	y = 4E+08x - 2E+07	0.9974	2.1
8-8nc-dFA	0.1-0.35	y = 4E+07x - 753238	0.9917	3.8
8-5nc-dFA	0.1-0.35	y = 5E+08x - 4E+07	0.9919	4.0
8-8THF-dFA	0.1-0.35	y = 6E+07x + 3E+06	0.9209	12.5
8-O-4-dFA	0.1-0.35	y = 2E+08x - 7E+06	0.9976	2.1
8-5c-dFA	0.1-0.35	y = 8E+06x + 257949	0.9603	8.9
5-5-dFA	0.1-0.35	y = 1E+08x - 3E+06	0.995	3.9
8-5dc-dFA	0.1-0.35	y = 1E+08x - 4E+06	0.999	1.5
8-8c/8-O-4-tFA	0.05-0.30	y = 7E+06x + 58679	0.9903	0.1
8-5nc/5-5-tFA	0.05-0.30	y = 2E+06x + 68547	0.9251	0.5
8-5nc/8-O-4-tFA	0.05-0.30	y = 1E+06x + 2447	0.8001	0.9
5-5/8-O-4-tFA	0.05-0.30	y = 795845x + 211746	0.9178	1.0

[a] c - cyclic form, nc - open (non-cyclic) form, THF - tetrahydrofuran form, dc - decarboxylated form; [b] coefficients of variation (CV) for the basic calibration (BC)